Vol. XVI, No. 6 1922

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

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CAMBRIDGE UNIVERSITY PRESS
C. F. CLAY, Manager
LONDON: Fetter Lane, E.C. 4
also
H. K. LEWIS & Co., Ltd., 136, Gower Street, London, W.C. 1
WHELDON AND WESLEY, Ltd., 2–4, Arthur Street, New Oxford Street, W.C. 2
CHICAGO: The University of Chicago Press
(Agent for the United States and Canada)
BOMBAY, CALCUTTA, MADRAS: Macmillan and Co., Ltd.
TOKYO: The Maruzen-Kabushiki-Kaisha

1922

Price Twenty Shillings net

PRINTED IN GREAT BRITAIN

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

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LXXIII. THE PRESENCE OF THE ANTINEURITIC AND ANTISCORBUTIC VITAMINS IN URINE.

By N. VAN DER WALLE.

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(*Received July 29th, 1922.*)

FUNK [1914], SEIDELL [1921] and others applied chemical methods in order to determine the structure of the antineuritic vitamin. Their efforts to isolate these mysterious substances were not perfectly successful and therefore we do not wonder that nothing is known as yet of the metabolism of these factors. However, recently some experiments have been made to examine the excretion of antineuritic vitamins from the organism.

Cooper [1914] succeeded in curing polyneuritis-pigeons with an alcoholic extract of faeces.

Muckenfuss [1918] tried to find antineuritic vitamins in ox-bile, human urine and saliva by shaking up these substances with fuller's earth. The fuller's earth, treated in this way, purified with water and alcohol and then dried, proved to be efficient in curing pigeons with polyneuritis.

Gaglio [1919] at Rome also investigated the result of administering urine to polyneuritis-pigeons. For this purpose he concentrated the urine and found that even 3–4 cc. were able to cure the birds. Urine of rabbits that had fasted for 15–20 days was likewise active, though less than that of normal rabbits which were fed on cabbage leaves and bran. The ash of urine proved to be without action.

Gaglio [1919] as well as Curatolo [1920] are convinced that these cures are not to be attributed to the action of vitamins but to the influence of non-specific products of metabolism. On this supposition is founded the research of di Matteï [1920], who tried to cure pigeons with coffee. Indeed, 5 % of mocha proved to have a strong curative power, but to his astonishment he perceived that pure caffeine (in non-toxic doses) had only a slight effect.

Funk [1912] had previously demonstrated that some purine and pyrimidine derivatives had a more or less favourable influence on polyneuritis.

It has also been proved that a mixture of NaCl and KCl may have a good, though temporary effect on the disease [Eijkman, van Hoogenhuyze and Derks, 1922].

Still more remarkable is the observation made by Theiler who described the favourable result of subcutaneous injection of distilled water [1921].

It is difficult to explain this effect from a stimulation of vitamins, still present in the organism. Theiler attributed this action to "*spontaneous recovery.*"

Jansen [1920], in the Medical Laboratory at Weltevreden (Java), confirmed the observations of Eijkman and Theiler.

Yet, the results described by Gaglio and Curatolo are so striking, that we doubt whether such a lasting and absolute recovery may be attributed to the influence of non-specific products of metabolism.

Funk informs us that the recovery obtained by treatment with hydantoin lasted only nine days, whereas a pigeon treated with thymus-nucleic acid lived on for a fortnight. Allantoin, adenine and other pyrimidine derivatives were less active. From the administration of uric acid he did not obtain any effect.

It occurs to us that much is to be said in favour of the opinion of Muckenfuss who believes antineuritic vitamins to be present in urine.

By a series of experiments on pigeons we tested the opinions of these investigators and tried to find the most probable explanation.

First, 12 pigeons (Nos. 1–12) were fed on polished rice and water till they showed symptoms of polyneuritis. Then 5 cc. of urine that had been concentrated *in vacuo* at 45° were administered *per os*. In six cases this had undoubtedly a favourable influence.

In most cases the symptoms had much improved one day after the administration; sometimes even the influence was evident after a few hours. The body weight, having diminished before the appearance of the clinical symptoms, went up again in all cases of improvement. Yet we did not succeed in regaining the initial weight.

Eight pigeons (Nos. 13–20) were treated prophylactically with the administration of concentrated urine from the very first. The result was that the average incubation period increased from 22 to 42 days.

To enhance, if possible, the curative power of the urine the latter was dried in large open Petri dishes in the incubator at 37°. After about 16 hours a thin layer of tough, semi-solid substance was found which was dried in an exsiccator at room temperature; 1 g. of dried substance was found to be equivalent to 25 cc. of urine with a S.G. of 1·030.

Seven polyneuritis-pigeons (Nos. 21–27) were treated with this dried urine, dissolved in autoclaved milk and administered in quantities of 1·5–2 g. per day. Four of these pigeons were cured rapidly and absolutely. After stopping the administration of dried urine, some days passed before the birds showed further symptoms of the disease. We succeeded in curing a second attack in two pigeons.

Three pigeons (Nos. 28–30) were treated by subcutaneous injection of dried urine, dissolved in sterilised milk. These however died after a few days.

It is well known that strong heating destroys the antineuritic vitamin. We therefore tried the action of urine which had been heated for one hour

at 130°. After sterilisation the urine was filtered, neutralised with hydrochloric acid and then concentrated *in vacuo* to a specific gravity of 1·050.

Ten polyneuritis-pigeons (Nos. 31–40) were treated with 15 cc. of this liquid daily. In one case the disease lasted only seven days, whereas the medicament had not the least effect on the other birds. This proves that by strong heating the curative power of the urine is decreased.

We also investigated the action of urine ash. For this purpose dried urine was incinerated, by which process it lost about 75 % of its weight. Ten pigeons (Nos. 45–54) were treated *per os* with this ash, suspended in water, and it was found to be almost inactive.

Chamberlain and Vedder [1911] observed that the antineuritic factor was removed by filtering through animal charcoal. We made use of this discovery and shook up urine with purified animal charcoal, 200 cc. of urine being shaken up for 45 minutes with 5 g. of animal charcoal; afterwards the urine was centrifuged and the charcoal shaken up anew with 200 cc. of fresh urine. This process was repeated three times. The charcoal was then washed once with distilled water and dried at 37°.

Six polyneuritis-pigeons (Nos. 60–65) were treated with this "*carbo cum urina*," 2 g. of this substance suspended in a little water being administered per day. All the birds recovered within a short time and we kept them healthy for at least a fortnight. The second attack too was cured. The smallest active dose proved to be 1 g.

Crude (non-purified) charcoal, shaken up with urine and treated in the same way, was far less active (Table I).

Table I. *Treatment of polyneuritis-pigeons with carbo cum urina.*

Laboratory no. of pigeon	Incubation period (days)	Period of illness* (days)	Effect of treatment	Dose of *Carbo cum urina*, g.	Remarks	Weight (g.) Beginning of experiment	Weight (g.) End of experiment
					Crude charcoal		
55	16	1	0	2	Died	240	200
56	21	2	0	2	,,	265	175
57	45	1	0	2	,,	240	175
58	22	5	±	2	Tempor. improved	350	225
59	16	12	±	2	,, ,,	260	200
					Carbo anim. puriss.		
60	24	18	+	2	Much improved	295	175
61	17	18	+	2	,, ,,	265	175
62	35	—	+	2	,, ,,	280	220
63	22	30	+	2	,, ,,	380	315
64	14	30	+	2	,, ,,	270	?
65	32	—	+	2	,, ,,	265	?
66	14	1	+	1	,, ,,	?	?
67	18	15	±	1	Slightly improved	355	260
68	17	19	±	0·5	,, ,,	330	290

* The period of illness is counted from the moment the treatment begins until death or, in case of recovery, until the end of experiment.

Another experiment on 12 pigeons (Nos. 69–80) showed that the non-treated animal charcoal had absolutely no effect.

Normal urine, shaken up with a surplus of charcoal, then filtered and concentrated *in vacuo*, had no effect (pigeons Nos. 81–89).

Then eight polyneuritis-pigeons (Nos. 90–98) were treated with animal charcoal, which had been shaken up with urine, heated previously for three hours at 120°. We expected this charcoal to be ineffective. On the contrary, two pigeons recovered and walked normally for ten days: six other pigeons were not cured. Without a doubt the curative effect of this charcoal is far inferior to that of charcoal shaken up with fresh urine. However, it must be admitted that there are some substances present in urine, possessing a curative power, and capable of being adsorbed by charcoal, and yet not bearing the character of the so-called vitamins. We tried to find out whether these substances have an organic or an inorganic character. For this purpose a mixture of inorganic salts was made, approximately in the same proportion as those found in human urine:

$NaCl$	9·2 %	Na_2HPO_4	5·0 %
K_2SO_4	2·7	$MgCl_2$	1·5
$(NH_4)_2SO_4$	1·5	$CaH_4(PO_4)_2$	0·25
KH_2PO_4	2·8		

This solution was shaken up with purified animal charcoal and ten pigeons (Nos. 99–108) were treated with this charcoal, which proved to have a very small effect as was expected beforehand, Rona and Michaëlis [1919] having demonstrated that animal charcoal will only slightly adsorb inorganic salts, the cations and anions present in urine being least adsorbed.

We may therefore safely assume that sterilised urine has a definite curative power owing to the presence of non-specific organic substances.

Finally we made an investigation as to whether the bacilli present in urine had any influence. The urine used was fresh, but not sterile. We did not expect to have a positive result here as the quantity of bacilli as far as weight was concerned, was very small.

Besides it has been already shown that *B. coli* does not contain any antineuritic vitamin even when cultivated in an extract of rice bran [Eijkman, van Hoogenhuyze and Derks, 1922], whilst Damon [1921] demonstrated that the B-vitamin is absent from *B. paratyphosus B.*, *B. coli* and *B. subtilis*. Fresh urine was kept at 37° for 24 hours. The bacilli out of this urine were cultivated on agar, then suspended in distilled water and afterwards heated at 100° for 30 minutes. This suspension of dead bacilli was then filtered through animal charcoal. After this process the latter was dried at 37°. As expected this charcoal proved to have scarcely any curative power (Table II).

Finally the fact that yeast is only active in a medium that contains antineuritic vitamin or its components was made use of [Eijkman, van Hoogenhuyze and Derks, 1922]. Baker's yeast was cultivated at 28° for 24 hours in fresh urine, the acid reaction of which had been diminished and to which had been added 5 % of glucose. This yeast was centrifuged, washed once with distilled water and dried at 37°. It proved to have a strong curative

action on polyneuritis of pigeons, 2 g. of yeast being sufficient to ensure the birds an absolute and lasting cure. This experiment makes it probable that antineuritic vitamins or their components are present in fresh, normal urine.

Table II. *Treatment with bacilli-charcoal.*

Laboratory no. of pigeon	Incubation period (days)	Period of illness (days)	Effect of treatment	Dose of bacilli-charcoal, g.	Remarks	Weight (g.) Beginning of experiment	Weight (g.) End of experiment
109	27	5	±	2	Slightly improved	330	230
110	31	6	±	2	„ „	360	350
111	19	4	0	2	Died	300	190
112	20	5	0	1	„	300	250
113	28	4	0	0·5	„	340	205
114	28	10	±	0·25	Slightly improved	350	200
115	15	7	±	0·25	„ „	325	260
116	19	4	0	0·25	Died	?	?
117	19	4	0	0·25	„	?	?
118	19	4	0	0·25	„	305	240

In order to trace whether diet has any influence on the activity of urine, the urine of a dog also was examined. Charcoal shaken up with this urine proved to possess as curative an action as human urine. Two g. per day were quite sufficient, but one pigeon even recovered from the use of 0·25 g. per day. As long as we were experimenting with its urine, the dog was fed on potatoes, polished rice and meat. This food contains a sufficient quantity of anti-neuritic vitamin. Then the diet was altered by autoclaving all the dog's food (bread, rice, meat and potatoes) during three hours. Only three weeks after the dog had been taking the vitaminless diet, the urine was again examined every day and shaken up with charcoal. The urine proved to have lost all its curative power (Table III).

Table III. *Treatment with charcoal shaken up with urine of a dog.*

A. The dog was fed on normal diet.

Laboratory no. of pigeon	Incubation period (days)	Period of illness (days)	Effect of treatment	Dose of charcoal, g.	Remarks	Weight (g.) Beginning of experiment	Weight (g.) End of experiment
123	13	—	+	2	Much improved	280	230
124	23	—	+	1·5	„ „	340	285
125	28	—	+	1	„ „	380	300
126	25	—	+	1	„ „	380	250
127	42	1	0	1	Died	300	195
128	33	8	±	0·5	Slightly improved	310	290
129	31	1	0	0·5	Died	320	190
130	37	—	+	0·25	Improved	290	245
131	35	8	0	0·25	Died	?	?

B. The dog was fed on a vitamin-free diet.

132	23	6	0	2	Died	320	260
133	28	2	0	2	„	300	220
134	22	4	0	2	„	315	200
135	21	2	0	2	„	360	?
136	26	5	0	2	„	330	?
137	22	6	±	2	Slightly improved	350	250
138	17	2	0	2	Died	300	240
139	26	5	0	2	„	340	205

It became more and more obvious that the favourable result must at least partly be attributed to the presence of the antineuritic vitamin, yet we went on to treat some fowls with urine because up till now it has never been demonstrated that non-specific substances have any influence on the polyneuritis of fowls. We found that fresh, concentrated urine (S.G. 1·050), as well as dried urine, when administered in large quantities is able to cure polyneuritis-fowls. Still more efficient was the action of animal charcoal, shaken up with fresh human urine. This however has to be administered in doses of 15 g. (equivalent to 2·5 litres of urine!) a day. Even then it required ten days to cure the fowl whilst we did not always succeed in curing the symptoms of paralysis absolutely. By a check experiment on six fowls it was shown that the charcoal itself did not exercise any curative action, and a fowl treated with charcoal that had been shaken up with urine, heated previously at 130° for one hour, did not recover.

The result of the experiments on fowls strengthened our opinion as to the presence of a small quantity of antineuritic vitamin in urine.

Antiscorbutic vitamin.

We then examined whether the antiscorbutic vitamin was also present in the urine.

For this purpose some guinea-pigs were put on a scorbutic diet receiving in addition fresh urine *per os*. The standard diet selected was a mixture of oats and bran, which together with water was given *ad lib*. Great care was taken to give the animals the best conditions possible. Following the example of Delf [Delf and Tozer, 1918] five guinea-pigs (Nos. 1–5) (Fig. 1) were given about 60 cc. of milk, previously autoclaved for an hour at 120°. Two other animals (Nos. 6 and 7), kept on the same diet, received daily 10 cc. of urine (which had been diluted to a specific gravity of 1·020). All these guinea-pigs developed scurvy and succumbed after some weeks. By post-mortem histological examination of the costochondral junctions we found the abnormalities described by Tozer [Delf and Tozer, 1918] in the severe stage of scurvy: irregularity of the junction, disorder of the rows of cartilaginous growing cells, usually a well-developed reticular zone, haemorrhages etc.

The average loss of weight of the guinea-pigs which were getting urine came to 29 % as against 19 % of the former group, the average lifetime being 28 days against 27 of the first group.

Evidently the administration of urine had no influence on the severity of the microscopical changes and on the average lifetime, whereas the loss of weight had increased.

(Though we made a histological examination of the ribs of all the guinea-pigs we are conscious that we have to be cautious in judging the results. It is known that Tozer [1921] demonstrated that the lack of A-vitamin may bring about departures from the normal which closely resemble those which are present in chronic cases of scurvy.)

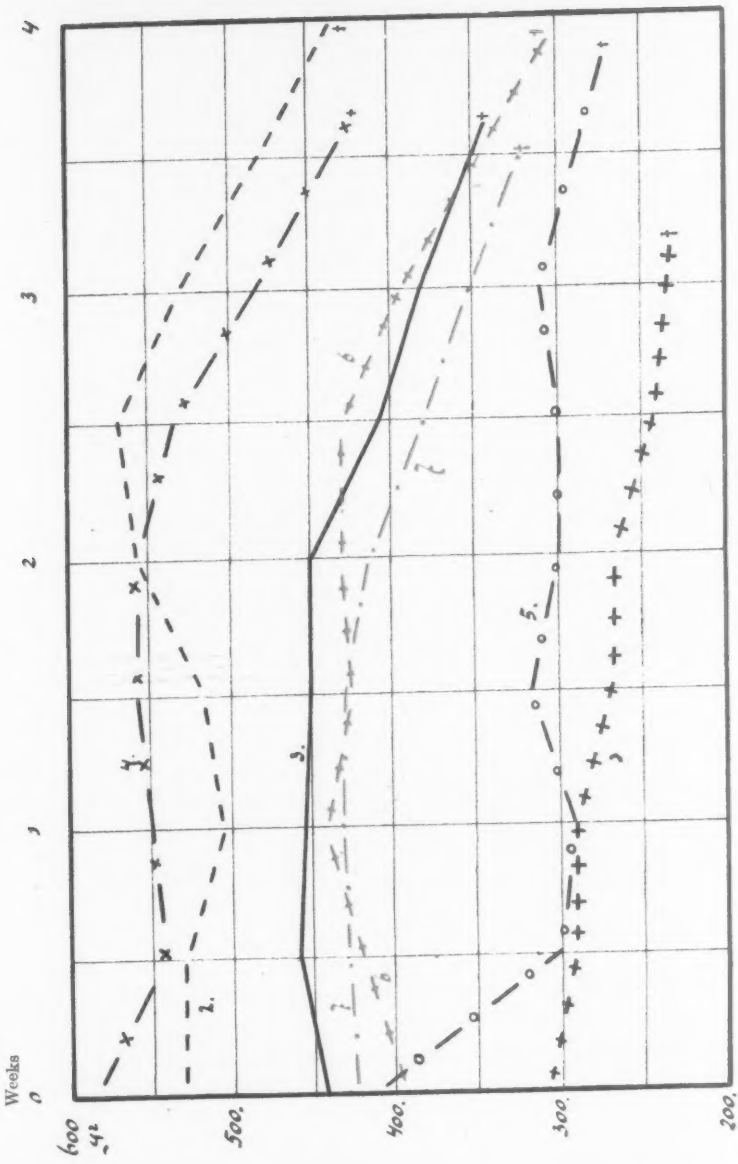

Fig. 1 Experiments I and I *a*.
Guinea-pigs, Nos. 1–5. *Diet*: oats, bran and water; autocl. milk (1 h. at 120°) *ad lib.*
Guinea-pigs, Nos. 6, 7. " " " " +10 cc. of urine.

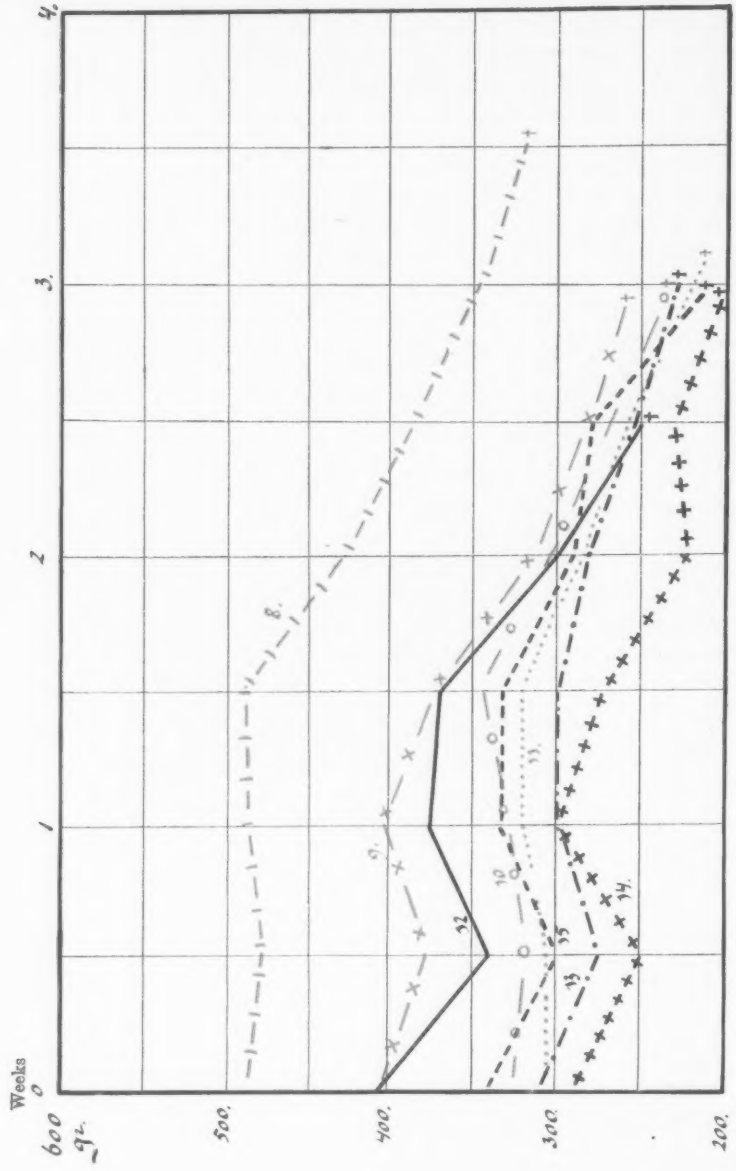

Fig. 2. Experiments II and II *a*.
Guinea-pigs, Nos. 12–15. *Diet:* oats, bran, water, 10 cc. of autocl. milk.
Guinea-pigs, Nos. 8–11. " " " " + 10 cc. of urine.

It appeared to us that the guinea-pigs were loth to take the autoclaved milk and it was necessary to give an additional quantity with a syringe, which took a great deal of time.

Experiment II. Four guinea-pigs (Nos. 12–15) were given with a syringe 10 cc. of autoclaved milk besides the usual scorbutic diet. Just as had been demonstrated by Delf, it was found that the loss of weight had increased (about 34 %). The average lifetime was about the same (25 days), and the microscopical changes were very similar in both experiments (Fig. 2).

Experiment II a. Another series of animals (Nos. 8–11), fed on the same standard diet, were also given 10 cc. of fresh urine daily. The average loss of body weight was 31 % and the average lifetime was 26 days. The microscopical abnormalities were not less than those in the above experiments.

Experiment II b. In this experiment the amount of urine was increased; 20 cc. being administered instead of 10. The average loss of weight of this group of animals (Nos. 16–19) was 26 %, and the average lifetime was 23 days (Fig. 3). On histological examination we found marked disorganisation of the normal structure. In this experiment too the urine proved to be without antiscorbutic power. (Although the administration of a large quantity of autoclaved milk had a favourable influence on the weight and the general state of the guinea-pigs, we thought it advisable to administer only 10 cc. of autoclaved milk to the animals in the following experiments, by which method it was still possible to make a comparison with the former groups.)

As we did not think it advisable to administer a greater dose of urine, we tried to find out in the following experiments whether urine was able to increase the antiscorbutic power of a small quantity of green cabbage.

Experiment III. Four guinea-pigs were given besides the diet of oats and bran 1 g. of green cabbage. These animals (Nos. 20–23) died in about 33 days, the average loss of weight being 32 %. The macroscopical as well as the microscopical changes were far less than in the former experiments (Fig. 4).

Experiment III a. 1 g. of green cabbage was evidently not quite enough absolutely to prevent the onset of scurvy. We then gave four guinea-pigs (Nos. 24–27) in addition 15 cc. of fresh urine. It was found that the average lifetime of these last animals, as compared to that of those in Expt. III, had increased only two days, the average loss of weight being 24 %. The result of the microscopical examination was similar to that of Group III. (We think the decrease of the average loss of body-weight was only owing to guinea-pig No. 23, the effect of the green cabbage being extraordinarily favourable in this case.) Without a doubt this experiment shows that the antiscorbutic power of 1 g. of green cabbage was great (a well-known fact), but that a dose of 15 cc. of urine was unable to exercise any curative effect.

Experiment IV. A further group of animals (Nos. 28–32) was given, besides the standard diet, 2 g. of green cabbage. By this treatment the average loss of weight was only 17 % whereas the average lifetime increased to 69 days. The influence of this quantity of cabbage proved to be much greater than the

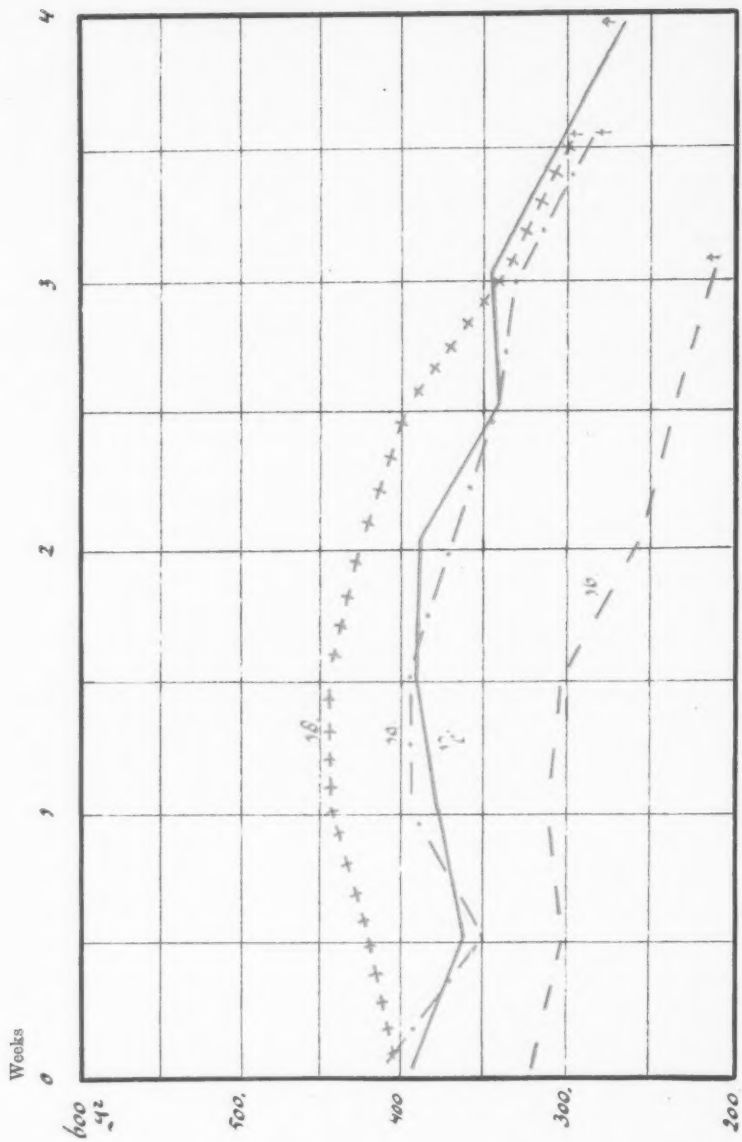

Fig. 3. Experiment II *b*.
Guinea-pigs, Nos. 16–19. *Diet*: oats, bran, water + 10 cc. of autocl. milk + 20 cc. of urine.

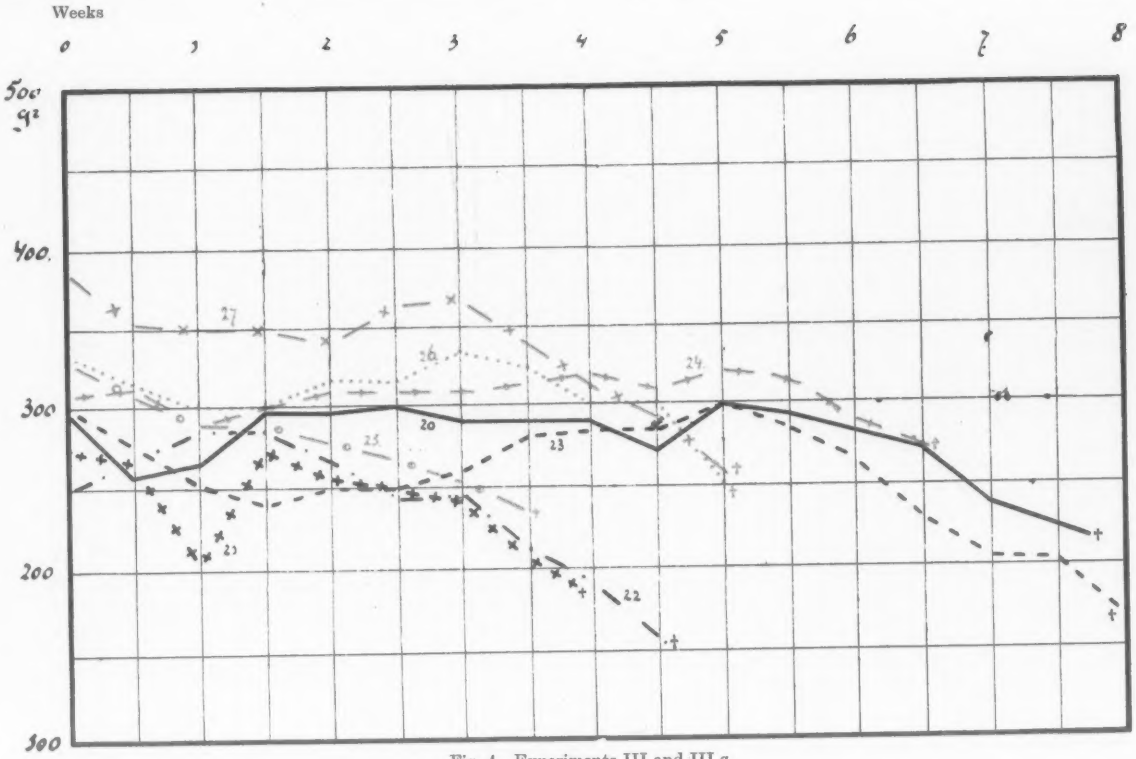

Fig. 4. Experiments III and III *a*.

Guinea-pigs, Nos. 20–22. *Diet*: oats, bran, water + 1 g. of green cabbage.
Guinea-pig, No. 23. „ „ „ + „ „ +10 cc. of autocl. milk.
Guinea-pigs, Nos. 24–27. „ „ „ + „ „ + „ „ +15 cc. of urine.

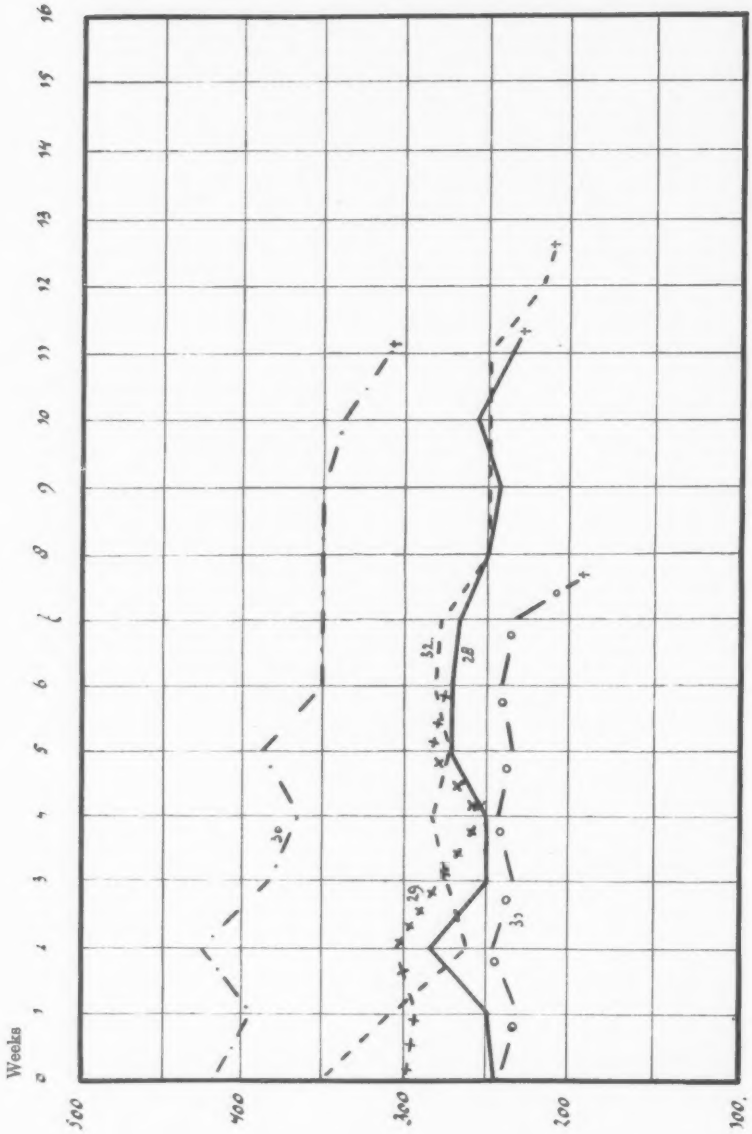

Fig. 5. Experiment IV.
Guinea-pigs, Nos. 28–31. *Diet*: oats, bran, water + 2 g. of green cabbage.
Guinea-pig, No. 32. " " " + " " + 10 cc. of autool. milk.

effect of giving a combination of 1 g. of cabbage plus 15 cc. of urine (Fig. 5). The microscopical changes found post-mortem were approximately those described by Tozer in the incipient stage.

In none of these experiments were we able to detect any antiscorbutic power of the urine. Of course it is possible that the urine might prove to be effective if given in greater quantities. This was practically impossible. Theoretically one might try to concentrate the urine just as Harden and Robison [1920] concentrated orange-juice and Bassett-Smith lemon-juice. We did not think it advisable to do this with urine because of the unknown influence of the other substances in urine on the labile antiscorbutic vitamins. Harden and Zilva [1918] demonstrated that charcoal does not adsorb the antiscorbutic vitamin and so we could not use this method of procedure, which proved so successful in the case of the antineuritic vitamin.

Conclusions.

1. It was shown that concentrated, normal urine may exercise a favourable influence on polyneuritis of pigeons.

2. Urine, dried at 37° for 16 hours, was likewise active.

3. By heating the urine at 130° for one hour, the curative power was destroyed.

4. The ash of urine was inactive.

5. *Carbo anim. puriss.*, shaken up with urine, centrifuged, washed with distilled water and then dried, had a strong curative power.

6. Urine, treated with a surplus of charcoal and then filtered, had lost its curative power.

7. Charcoal, shaken up with urine, which had been heated previously for three hours at 120°, was far less active.

8. Charcoal not shaken up with urine proved to be quite inactive.

9. Charcoal, shaken up with a solution of inorganic salts, containing the cations and anions present in urine, had only a slight effect.

10. The curative power exercised by urine may not be attributed to the presence of bacilli.

11. Yeast, cultivated in urine, to which had been added 5 % of glucose, exercised a strong curative action.

12. The urine of a dog had as strongly curative an action as that of man, but when the dog was fed on a vitamin-free diet, the urine lost its curative power.

13. It was shown that in some cases fresh, normal urine or dried urine, when given in large quantities, may have a good influence on polyneuritis of fowls.

14. Charcoal shaken up with normal urine was likewise active.

15. The charcoal itself had no curative influence on the polyneuritis of fowls.

16. Charcoal treated with urine heated previously for one hour at 130° had no effect.

17. It was pointed out that a small quantity of antineuritic vitamin is present in the urine, as proved by the above experiments.

18. We were unable to detect the presence of the antiscorbutic vitamin in urine.

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LXXIV. THE SUCTION PRESSURE OF THE PLANT CELL.

A NOTE ON NOMENCLATURE.

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(*Received August 4th, 1922.*)

As a result of the work of Pfeffer and de Vries during the last thirty years of the nineteenth century, the vacuolated cell came to be regarded as a system consisting of a solution containing osmotically active substances (the cell sap) surrounded by a semi-permeable membrane (the protoplasm) permeable to water but impermeable to the solutes in the cell sap, the whole being enclosed by an elastic cellulose envelope (the cell wall), supposed to be permeable both to water and dissolved substances. How far such a view really represents the facts is not in question here.

When such a system is immersed in water, it is to be expected that water will enter the cell on account of the osmotic pressure of the cell sap. This must result in the development of a hydrostatic pressure and consequent stretching of the wall, which, being elastic, exerts an inwardly directed pressure on the cell contents. At any time, therefore, there is a pressure, the osmotic pressure, tending to send water into the cell, and the inwardly directed component of the pressure exerted by the wall tending to compress the cell contents and so send water out. The hydrostatic pressure of the water in the cell is called the turgor pressure, to which the inwardly directed wall pressure must be equal and opposite. If this pressure is denoted by T and the osmotic pressure by P, the net pressure sending water into the cell when this is surrounded by water is thus $P - T$, which varies from P when the cell is completely deturgid to zero when it has absorbed so much water that $T = P$ and the cell can be regarded as saturated with water.

Just as it is convenient to have the term osmotic pressure to denote the pressure developed when a solution is separated from pure water by a semi-permeable membrane, so it is convenient to have a term to denote the actual net pressure sending water into the cell when this is immersed in pure water. For this quantity Ursprung and Blum [1916] have used the term "suction force" (Saugkraft) and there appears to be a tendency for other continental writers to adopt this. The chief objection to this term is that the quantity is a pressure and not a force, and is equated with a pressure (osmotic pressure). In this country Thoday [1918] has proposed the term "water absorbing power"

of the cell for this quantity. This term appears particularly unfortunate, for not only is it perhaps a trifle clumsy, but if the quantity in question is not a force, still less is it a power, a rate of doing work. Nevertheless there is a tendency for the term to be accepted by English writers, one of whom indeed gives an equation in which a quantity defined as a surface tension (a force per unit length) is put equal to a quantity defined as a pressure (a force per unit area) less a quantity defined as a power (work per unit time).

To avoid such anomalies in nomenclature, I propose for this quantity, already described as a force and a power, but which is in reality a pressure, the term "suction pressure." This gives an accurate description of the quantity, is not clumsy, and practically embodies the term used by Ursprung and Blum, whose expression, other things being equal, is entitled to precedence on the ground of priority.

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LXXV. RESPIRATORY EXCHANGE IN FRESH WATER FISH.

PART IV.

FURTHER COMPARISON OF GOLD-FISH AND TROUT.

By JOHN ADDYMAN GARDNER and GEORGE KING.

From the Physiological Laboratory, University of London, South Kensington.

(*Received August 21st, 1922.*)

In Part II of this series of papers [1914, 2] the behaviour of trout at various temperatures under reduced oxygen tension was described, and measurements were recorded of the quantity of oxygen in the water at the asphyxial point for the various temperatures. It was found that the oxygen content of the water at the asphyxial point was much larger at higher temperatures than at lower, a result to be expected since, as was shown in Part I [1914, 1], the oxygen consumption of trout in fully saturated water increases with the rise in temperature—within the ordinary limits of temperature of natural water consumption doubles for rise of 10°—and consequently the fish would have to pump a much larger volume of water through their gills at a higher temperature than at a lower to obtain the necessary amount of oxygen.

Gold-fish as we showed in Part III [1922] require much less oxygen than trout and live at an altogether lower plane of metabolism, though their oxygen requirements are also proportional to temperature. It seemed of interest to measure the oxygen tension at the asphyxial point at various temperatures in the case of these animals.

Method.

The apparatus described in Part II proved unsuitable for gold-fish, as we found it impossible to reduce the oxygen in the water to the asphyxial tension within a reasonable time. We therefore used a much smaller apparatus based on the same principle, and filled with water which had previously been heated in order to get rid of the bulk of the dissolved gas and allowed to cool in a closed bottle. A six litre vacuum desiccator with slightly domed lid served for the water tank. This was filled to the edge of the ground flange with the water so that the gas space above the water was only that of the shallow dome of the lid. The tubulure of the lid was closed by a rubber stopper carrying (1) a tube leading to the bottom of the vessel and ending in a perforated bulb through which nitrogen could be sprayed through the water, (2) an exit tube for nitrogen bent over so as to dip into a beaker of water

and (3) a capillary tube fitted with a tap through which samples of the water could be drawn off as required. The desiccator stood in a thermostat. The fish were placed in the tank and the air in the water was gradually displaced by a current of nitrogen, which also served to stir the water. The compressed commercial nitrogen used contained about 0·5 % of oxygen; this quantity of oxygen was too much, and the gas was purified by passing through several wash bottles containing strong alkaline pyrogallol. In this way the percentage of oxygen in the nitrogen could easily be reduced to 0·14. A sample of the water was withdrawn for analysis at the asphyxial point, which was taken as that point at which the fish rolled over on their sides and ceased to breathe. The sample was withdrawn by displacement of mercury and analysed by the method fully described in previous papers.

Between temperatures of, say, 10° and 27° the oxygen content of the water could fairly easily be reduced to the asphyxial tension by means of a current of nitrogen, but at low temperatures it was not found possible to reduce the oxygen sufficiently by this method. In experiments at low temperatures the oxygen was displaced as far as possible by a current of nitrogen, and the apparatus was then closed and the fish made to use up the remaining oxygen themselves.

Experimental.

Experiment at high temperature.

Two fish, *A* weighing 53 g. and *B* weighing 58 g., were placed in the partially boiled out water at a temperature of about 22° and the tank put in the thermostat at 28°; nitrogen was then bubbled through the water. At the beginning the fish were very active, *A* being particularly violent for a gold-fish, and attempting to jump out of the water. The respirations of both animals were about 117 per minute. As the oxygen tension decreased the respiration became more spasmodic, irregular and slow. The movements of the fish gradually became less vigorous, and towards the end of the experiment the animals took short intervals of rest with scarcely any perceptible movements of the gills, alternating with short rushes round the vessel mainly near the surface. After about 90 minutes *A* remained on his side without any obvious sign of life and *B* was nearly in the same condition. A sample of water was now withdrawn for analysis, the temperature being 27·2°. The fish were now removed from the experimental vessel and placed in a trough of tap water at laboratory temperature. After two minutes *B* recovered his normal position, while *A* began to breathe and showed slight movements after five minutes, but still remained on his side. The fish were then placed in the store tank in running water and after two to three hours *A* recovered. Next day both fish appeared to be perfectly normal and healthy. On analysis 1 litre of the water was found to contain the following quantities of gas measured at 0° and 760:—free and combined CO_2 12·25 cc., oxygen 0·422 cc. and nitrogen

8·287 cc. Taking Winkler's value 0·02727 as the absorption coefficient of oxygen at 27·2° (Landolt, Börnstein, Meyerhoffer's tables) the partial pressure of the dissolved oxygen was therefore 1·547 % of one atmosphere.

Experiment at temperature 11–12°.

Two fish, *C* 51 g. and *D* 40 g., were placed in six litres of partially boiled out water as before and purified nitrogen bubbled through the water. Both animals were fairly active at first and their respirations 80–90 per minute, the normal in fully aerated still water being between 40–50 per minute. During the first two hours of the experiment, as the oxygen gradually decreased, the fish became less continuously active, the number of respirations decreased to 40–50 per minute, and the breathing, if we can use this term, became shallower. A noticeable feature was that under these low oxygen tensions the fish indulged in spells of backward swimming. After 135 minutes the nitrogen current was stopped and the fish reduced the oxygen content of the water naturally. The breathing became shallow and very spasmodic and the respiration rate was reduced to 20–30 per minute. At the end of the fourth hour it was noticed that the fish remained inert for periods of 1–1½ minutes. This was followed by a few respirations then another period of inertness and so on. After six hours both fish were on their sides and seemed to have ceased to respire, a sample of water was therefore withdrawn for analysis. The temperature was 11·4°. One litre of the water contained 10·732 cc. free and combined CO_2, 0·397 cc. oxygen and 15·362 cc. nitrogen measured at 0° and 760. Taking the coefficient of absorption for oxygen in water at 11·4° as 0·03686, the partial pressure of the oxygen was 1·077 % of one atmosphere. The fish gradually recovered on being restored to the store tank.

Experiment at low temperature.

It was not found possible at the low temperature of the ice chest to reduce the oxygen tension sufficiently, by means of a current of nitrogen, to incommode, let alone asphyxiate, the fish. After reducing the tension as far as possible by this method we had to rely on the fish to use up the remaining oxygen, and this proved a very slow process.

Two fish, *E* 52 g. and *F* 42 g., were placed in 5½ litres of boiled out water and a rapid current of nitrogen bubbled through for two hours on March 27th. The nitrogen inlet and outlet taps were then closed and the vessel placed in the ice chest and left for a week. On the second day the fish appeared to be quite normal and during the week they remained very quiet and sluggish sometimes near the surface and sometimes at the bottom of the vessel. On April 5th they showed a rolling gait symptomatic of distress. On the morning of April 6th *F* was more or less on his side and in the afternoon appeared to be dead or nearly so. *E* was still breathing occasionally in a spasmodic manner and had a rolling gait as though finding difficulty in keeping upright. The temperature recorded by the thermometer in the upper part of the chest

varied from 3–5° during the week. A sample of water was withdrawn for analysis at 2.35 p.m. on April 6th; its temperature was 2·6°. The quantity of oxygen in the water was less than the limit of the experimental error. On analysis one litre of water was found to contain the following volumes of gas reduced to 0° and 760:—free and combined CO_2 96·96 cc., oxygen nil, nitrogen 22·97 cc.

Comparison with trout.

Trout normally use very much larger quantities of oxygen per kilo per minute than gold-fish, and at a diminishing oxygen tension behave quite differently. The behaviour of trout was described in detail in Part II [1914, 2, p. 595]. As the oxygen diminished the respiratory movement gradually increased in rapidity and force and acquired a dyspnoeic character. The animals became more active, rushing about and jumping into the air space above the water. These periods of activity alternated with periods of rest at the bottom with the dyspnoeic respiration mentioned. The periods of rest gradually became longer than the periods of activity, and as the oxygen approached the asphyxial tension they remained nearly all the time at the bottom and ultimately quietly rolled over on their backs. In this condition they died in two or three minutes, but if oxygen were at once sprayed into the water they recovered in the course of a few minutes and afterwards appeared to suffer no ill effects. The asphyxial condition in the case of trout takes place within quite small limits of oxygen tension; at or below this tension they very quickly die but slightly above they recover. Gold-fish on the other hand could stand much lower oxygen tensions for any given temperature and are much less sensitive, so that the precise asphyxial tension is difficult to ascertain. This difference is still more marked at low temperatures. In Table I we give a comparison of the measurements for trout and gold-fish.

Table I.

	8″ trout, weight 80-90 g. Oxygen in water at asphyxial point*		5″ to 6″ gold-fish, weight 45-55 g. Oxygen in water at asphyxial point		Normal water	
Temperature	cc. of oxygen measured at 0° and 760 per litre of water	partial pressure of oxygen in per cent. of 1 atmosphere	cc. of oxygen measured at 0° and 760 per litre of water	partial pressure of oxygen in per cent. of 1 atmosphere	cc. of oxygen at 0° and 760 per litre	partial pressure in per cent. of 1 atmosphere
2·6°	—	—	nil	nil	9·49	
6·4°	0·79	1·89	—	—	8·60	
9·5 to 10°	0·81	2·19	—	—	8·00	
11·4°	—	—	0·397	1·077	7·9	
17°	1·37	4·17	—	—	6·75	app. 21 %
18°	1·49	4·62	—	—	6·61	
24°	1·97	6·83	—	—	5·89	
25°	2·40	8·49	—	—	5·78	
27·2°	—	—	0·422	1·547	5·52	

* Gardner and Leetham [1914, 2].

Glycogen content of trout and gold-fish under various conditions.

In Part I [1914, 1] of this series it was shown that at low temperature, *i.e.* below about 6°, trout appeared to be in a condition, for a time at any rate, akin to that of hibernating animals, and their respiratory quotients were very low. These low respiratory quotients could not be directly connected with nitrogen metabolism, and it was suggested that a possible explanation might be found in the fat. If at low temperature the animals were in a state of hibernation or starvation they might be living on their fat and partially converting it into glycogen and sugar. Some such process might result in a low respiratory quotient, as indicated in the following equation:

$$2C_3H_5(C_{18}H_{33}O_2)_3 + 64O_2 = 16C_6H_{12}O_6 + 18CO_2 + 8H_2O$$

$$CO_2/O_2 = \tfrac{18}{64} = 0{\cdot}281.$$

In the hope of throwing light on this point comparative estimations of the glycogen content of starving trout at various temperatures were made. The glycogen was estimated by Pflüger's method. The precipitated glycogen, after solution and reprecipitation two or three times, was hydrolysed and the sugar estimated by Fehling. If in sufficient quantity the cuprous oxide was weighed, otherwise it was dissolved in ferric sulphate and the ferrous salt produced estimated by permanganate. The first experiments were done on four-inch trout which however proved too small for the purpose.

Experimental.

Experiments with four-inch trout.

(1) *Control.* Four fish weighing 44·65 g. were kept in running water in a large tank with a gravel bottom. The average temperature of the water was 19°. The fish were not fed though they may have picked up some food. After treatment by Pflüger's method and hydrolysis of the glycogen 0·0013 g. Cu_2O was obtained. Percentage of glycogen was therefore 0·0052.

(2) Four fish weighing 40·6 g. were placed in an open vessel in the ice chest and the water kept well oxygenated by a spray of oxygen. At the end of the first day the temperature of the water was 7°, at the end of the second day 6° and the third day below 5°. The fish were very inert and sluggish and scarcely moved when touched. On the afternoon of the third day they were killed and analysed. 0·0032 % of glycogen was found.

(3) Five fish weighing 57·5 g. were kept in the ice chest under the same conditions as in experiment 2 for six days. On the last day two died and as soon as this was observed the rest were killed and the whole analysed. The final temperature of the water was 3·5°. The percentage content was 0·0022.

(4) Three fish weighing 27·8 g. were kept in the ice chest for two days at reduced oxygen tension. They were then analysed but no glycogen could be detected.

As the fish were too small to give a suitable weight of material without using an inconvenient number of fish the experiments were repeated using eight-inch animals.

Experiments with eight-inch trout.

(5) *Control experiments.* The fish were kept in a large outside tank in running water for several days. They were not fed and the temperature of the water was about 16°. On analysis, fish *A* weighing 89·1 g. gave no trace of glycogen, fish *B* weighing 109 g. was found to contain 0·0012 %.

(6) Two fish were placed in separate earthenware bowls in the ice chest and a spray of oxygen passed through the water by means of circular lead pipes punctured with small holes. The fish were kept at 2·6° for two days; they were very sluggish but otherwise appeared healthy. Fish *C* weighing 114·3 g. contained no trace of glycogen nor did fish *D* weighing 134·3 g.

(7) Two fish were kept under similar conditions to those in experiment (6) for five days. Final temperature of the water was 1·7°. On the fifth day one of the animals turned over and they were therefore cut up and analysed. Fish *E* weighing 101·3 g. gave 0·1179 g. of Cu_2O and contained 0·0469 % glycogen. Fish *F* weighing 108 g. gave 0·0798 g. Cu_2O and hence contained 0·0294 % glycogen.

We do not know what is the limit of accuracy of Pflüger's method for estimating glycogen, but as the conditions—proportion of alkali, duration of hydrolysis, volume of alcohol—were kept as nearly as possible the same in experiments 5, 6 and 7 it is clear that the fish in experiment 7 contained a very much larger percentage of glycogen than the controls. This result seems to fit in with our hypothesis as to the cause of the low respiratory quotients at low temperature. At the same time we cannot pretend that the experiments are altogether conclusive.

GLYCOGEN CONTENT OF GOLD-FISH.

It seemed of interest to ascertain how glycogen content of gold-fish, which did not show abnormal quotients at low temperatures, compared with that of trout.

(8) *Control experiments.* Two gold-fish were taken from the running water tank in which they had been living many months. The tank contained plenty of food. Two fish weighing 73 g. analysed together were found to contain 0·86 % of glycogen. Temperature of the tank when the fish were caught 13–14°. Two fish weighing 90·5 g. taken from water at about 16° contained 1·57 %. Two fish weighing 61·2 g., temperature of tank 18°, contained 1·235 % glycogen.

(9) Two fish weighing together 73·3 g. were kept in an open vessel in the ice chest for seven days; the water was aerated by a current of oxygen and the final temperature was 2–3°. The fish were not fed, and on analysis they contained 1·21 %.

(10) In this experiment two fish weighing 81·8 g. were placed in the apparatus used for determining the asphyxial point and the oxygen tension considerably reduced by bubbling nitrogen through the water. The apparatus

was then placed in the ice chest and kept at about 2° for seven days. Percentage of glycogen was found to be 0·52.

(11) Two fish total weight 61·6 g. were placed in a trough of ordinary tap water through which oxygen was bubbled occasionally and kept in the ice chest for eleven days; the fish were not fed. Percentage of glycogen was found to be 0·54.

(12) Two fish weighing 60·45 g. were placed in six litres of partially boiled out tap water in the asphyxial point apparatus referred to above. Pure nitrogen was passed through the water for half an hour when the nitrogen tubes were closed and the apparatus placed in the ice chest and left for several days. The average temperature of the water was 3·7°. On the morning of the fifth day one fish was found on its side but the others were still alive and in normal position. They were killed and the whole analysed and found to contain 0·024 % glycogen.

These results appear to be in conformity with the low level of metabolism of these fish compared with trout.

We take this opportunity of expressing our thanks to the Government Grants Committee of the Royal Society for aid in carrying out this work; and also to Mr A. R. Peart of Hungerford who supplied the trout.

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LXXVI. RESPIRATORY EXCHANGE IN FRESH WATER FISH.

PART V. ON EELS.

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(*Received August 25th, 1922.*)

Eels are said to be peculiarly averse to cold, and the fact that the temperature of the brackish water of estuaries is usually higher than that of unmixed salt or fresh water has been advanced as one of the reasons for their seaward migration on the approach of winter. During the cold of winter these fish lose their appetite and become torpid, large numbers of them congregating together for the sake of the additional warmth thus obtained, and burying themselves to a depth of many inches in places where the receding tide leaves them more or less dry.

In the eel the bronchial openings are small and lead into a sac, from which another sac is given off. The gills are thus exposed only slightly to the drying influence of the atmosphere, and it is believed that it is owing to this and to the slimy condition of the skin, that eels are able to exist for a very considerable time, compared with other fish, out of water. They can evidently exist at a low plane of metabolism, but like most animals which pass the winter in a torpid condition, are relatively voracious during the summer months.

For these reasons it seemed of interest to study the respiratory exchange of these animals under various conditions of temperature, and compare them with other fish, particularly trout, which, as we have shown, are also sluggish, for a time at any rate, on exposure to cold.

The fish used in these experiments were the common *Anguilla vulgaris* and were 15 to 18 inches in length. They were kept for several days in a large tank with gravel bottom in running water before being used for experiment. The tank contained a number of small worms and the like, which the eels could have picked out of the gravel, but whether they did so we have no means of judging. The fish however were in good condition.

The method adopted was that fully described in Part I [1914] and Part III [1922] of this series.

Experimental.

At high temperatures.

(1) Six fish, weight 940 g., were placed in a tank of water at the temperature of the outside tank—about 15°—and gradually warmed up to 20° before being placed in the experimental bottle. The initial temperature of the water in the bottle was 21·1° and the final temperature 23·5°; the duration of the experiment was 5 hours 4 minutes, and during this period 275 cc. of commercial oxygen were added (measured at 16·5° and 760 mm.) to the air above the water. Analyses of the air and water at the beginning and end of the experiment gave the following results in cc. reduced to standard temperature and pressure.

	Initial vol.	Final vol.	Difference
Free and combined CO_2	1827·4	2023·0	+195·6
Oxygen	4102·3	3810·2	−292·1
Nitrogen	14693·1	14721·4	+ 28·3

Nitrogen error +0·1990 %.

At the end of the experiment the water (24619 cc.) had a strong faecal smell, and contained a good deal of dejecta. The eels at this high temperature were very active and difficult to catch by hand.

At medium temperatures.

(2) Five fish, weight 650 g. Initial temperature of water 16·5°, final temperature 16·5°. Duration of experiment 5 hours 45 minutes. Oxygen was added as before. The fish were lively.

	Initial vol.	Final vol.	Difference
Free and combined CO_2	1821·0	1950·1	+129·1
Oxygen	4147·5	3981·5	−166·0
Nitrogen	15982·2	15981·8	− 0·4

Nitrogen error −0·0025 %.

(3) Five fish; weight 725 g. Initial temperature 11·7°, final temperature 8·4°. Duration 3·44 p.m. to 12.10 p.m. = 20 hours 36 minutes. Oxygen was added, and pumping was continuous except for a few hours owing to a slipped belt.

	Initial vol.	Final vol.	Difference
Free and combined CO_2	1552·9	1828·8	+275·9
Oxygen	4868·8	4548·4	−320·4
Nitrogen	17594·7	17607·8	+ 13·1

Nitrogen error +0·074 %.

(4) Four fish, weight 680 g. This experiment was commenced at 11.37 a.m. and continued until 12 noon on the following day—24 hours 23 minutes. The initial temperature of the water in bottle was 16·6° and the air 16·7°. After about three hours the water in the thermostat was packed with ice, and the temperature of the thermometer in the air of the bottle was noted at intervals, the final temperature of the water was 6·4°. It was estimated

that the average temperature was between 9° and 10°. Oxygen was added during the experiment as before. During the night the pumping was interrupted for a few hours owing to the slipping of a belt, probably in the early morning owing to change over of the dynamos in the power station.

	Initial vol.	Final vol.	Difference
Free and combined CO_2	1650·1	1949·8	+299·7
Oxygen	4655·4	4371·7	−283·7
Nitrogen	16875·8	16903·4	+ 27·6

Nitrogen error +0·16 %.

At low temperatures.

(5) For this purpose the eels were kept in well oxygenated water in the ice chest for 24 hours before the experiment, the average temperature being between 3° and 4°. The animals were very inert and sluggish and scarcely moved when handled. Five fish, weight 725 g. Initial temperature of water 6·5°, final temperature 5·3°. Duration of experiment 24 hours 8 minutes. Oxygen was added during the experiment and the pumping was continuous.

	Initial vol.	Final vol.	Difference
Free and combined CO_2	1343·5	1414·3	+ 70·8
Oxygen	5559·5	5397·2	−162·3
Nitrogen	20211·6	20211·5	− 0·1

Nitrogen error −0·00049 %.

In all the above experiments except that at high temperatures the water remained clear and no unpleasant smell was noticed. The fish remained perfectly healthy after the experiments. The results are summarised in the following table.

No. of experiment	Average temperature	Average weight of fish used in g.	Oxygen absorbed per fish per hour in cc. at 0° and 760	Oxygen absorbed per kg. per hour at 0° and 760	CO_2 evolved per fish per hour at 0° and 760	CO_2 evolved per kg. per hour at 0° and 760	Respiratory quotient
(1)	22·2°	157	9·60	61·29	6·43	41·04	0·67
(2)	16·5°	130	5·77	44·41	4·49	34·54	0·78
(3)	10°	145	3·11	21·45	2·68	18·47	0·86
(4)	about 9°	170	2·91	17·11	3·07	18·08	1·06
(5)	5·9°	145	1·35	9·28	0·59	4·05	0·44

It will be noticed that the oxygen consumed per fish, and per kg. of fish per hour is proportional to the temperature and if the oxygen is plotted against temperature the values are approximately on a straight line.

On comparison with the figures given in Part I [1914] for 8-inch brown trout it is evident that eels live at a much lower plane of metabolism than trout. At medium temperatures, about 16°, trout use about four times as much oxygen as eels, and at low temperatures 10 to 12 times as much.

The respiratory quotients in the first four experiments are approximately normal but at low temperature, at which the animals are in a torpid condition, the value falls much below normal. In this respect their behaviour recalls that of trout.

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LXXVII. ON A SERIES OF METALLO-CYSTEÏN DERIVATIVES. I.

By LESLIE JULIUS HARRIS.

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(*Report to the Committee of Scientific and Industrial Research.*)

(*Received September 14th, 1922.*)

When a stream of air is blown through an alkaline solution of cysteïn, the oxidation is generally accompanied by the production of a violet coloration; an observation that will be familiar to workers who have had occasion to prepare cystine in this manner. Although the phenomenon has been long known its cause has remained obscure, and the present investigation was undertaken primarily with the object of ascertaining its nature.

The fact that cysteïn gives with ferric chloride and ammonia an intense coloration, which becomes darker on shaking, was first noticed by Andreasch [1884]. Arnold [1911], describing a number of tests by which cysteïn may be recognised, distinguishes between the colour produced with ferric chloride and that with ammonia. "If a solution of cysteïn is made weakly alkaline a violet colour is produced which is more permanent than that given by sodium nitroprusside...." There can be little doubt however that the coloration is in reality due not so much to the action of the alkali upon the cysteïn, as would appear from Arnold's account, as to the presence of traces of a metallic (generally ferric) salt, which produce the typical coloration in alkaline solution. Taking care to ensure the complete absence of a metallic ion I have been unable to observe the production of the slightest colour.

An important feature of the reaction is its impermanent character. Arnold observes that the colour fades after a while, but can be renewed by shaking. The nature of this curious alternation of fading and restoration of colour receives no explanation from Arnold.

The Reversible System: Cysteïn—Iron—Ammonia.

When cystine is isolated by the use of mercuric sulphate reagent, the Hg-cystine compound which is precipitated, yields, on decomposition with H_2S, a solution of the reduced cysteïn. This can be re-oxidised, after removal of H_2S, by making alkaline with ammonia and blowing a stream of air through the solution. Under these circumstances there is generally enough iron present

as impurity to produce a noticeable violet coloration in the solution as soon as the active oxidation is started. Often a state of delicate equilibrium is set up in which the colour disappears upon the air current being stopped, and immediately reappears upon its continuation. The necessary conditions may be reproduced artificially by adding to a test tube containing (say) 4 cc. of a 5 % cysteïn solution a single drop of weak $FeCl_3$ solution. Upon making alkaline with weak NH_4OH, a violet colour appears, only to fade almost immediately. The colour reappears if the tube be shaken, or if air (or, better, oxygen) be otherwise brought into intimate contact with the solution. Bubbling hydrogen, carbon dioxide or nitrogen through the liquid has no effect.

Variation in the concentrations has a visible effect on the course of the phenomena. The depth of colour produced is directly proportional to the quantities of both cysteïn and ferric chloride present together. Again, the greater the concentration of ferric chloride relative to that of cysteïn, the longer does the violet colour survive the cessation of the air current, or, to look at it in another way, increasing the proportion of cysteïn in the mixture, brings about a more rapid fading when the air current is stopped. The solution remains violet (or alternatively capable of yielding a violet colour on aeration) so long as unoxidised cysteïn is present; the presence of the latter can be demonstrated by the extremely delicate nitroprusside test[1]. When oxidation is complete the solution consists of cystine together with a minute quantity of $Fe(OH)_3$, derived from the added iron salt. The effect of increasing the alkalinity is to yield a solution which less readily decolorises in the absence of the air current, but in which complete and permanent loss of colour (or ability to produce it on addition of the iron salt) occurs more rapidly, owing apparently to the greater rapidity of the transformation cysteïn $\longrightarrow$ cystine in the more alkaline solution.

Numerous ferric salts, also suspended ferric hydroxide, have been investigated as substitutes for $FeCl_3$. All were equally efficient. Potassium ferricyanide was without action.

Explanation of the Phenomena.

Several explanations might be adopted to account for the observations described above. At first sight it would seem likely that the colour is due to the formation of an unstable compound in a state of oxidation intermediate between cysteïn and cystine; its presence being made manifest as a result of its power to yield a coloration with ferric salts.

Secondly, it may be assumed that direct oxidation of cysteïn to a body of the cystine peroxide type occurs, and that this hypothetical substance is reactive to $FeCl_3$. The consecutive reaction is between cysteïn and the peroxide to yield the end product, cystine. There are theoretical grounds for postulating the possible existence of such a peroxide.

[1] It is possible to fix approximately the completion of oxidation by the disappearance of the violet coloration.

In view, however, of similar reactions between cysteïn and salts of certain other metals, notably copper, the following explanation would appear to fit the facts more closely than either of these.

Cysteïn is an acid, and it may be supposed that in the presence of $Fe^{\cdots}$ ions, and in alkaline solution, it forms a violet-coloured ferric derivative. The main characteristic of cysteïn is its reducing power:

$$2COOH—CH(NH_2)—CH_2—S\boxed{H+O} \longrightarrow (COOH—CH(NH_2)—CH_2—S—)_2+H_2O.$$

If excess is present and the supply of air be limited the cysteïn is oxidised to cystine at the expense of the ferric ion, which is thereby reduced to the ferrous state. The latter yields no coloured derivative with cysteïn, and the solution therefore fades. If now air be blown through the mixture, the $Fe^{\cdot\cdot}$ is rapidly re-oxidised to $Fe^{\cdots}$, $Fe^{\cdot\cdot}$ as is well known being unstable in alkaline solution when available oxygen is present. The colour is therefore regenerated provided some unoxidised cysteïn remains to form the anion of the coloured body, and it will be retained until the process of oxidation is complete. In the presence of abundance of free oxygen the cysteïn will be oxidising continuously, and the iron will be maintained in the ferric condition.

This explanation accounts for the following experimental observations:

(1) The intensity of colour on making alkaline with ammonia is proportional to the amounts of cysteïn and Fe salt present together (*i.e.* the amount of cysteïn iron derivative produced).

(2) Excess of cysteïn causes a more transient coloration (since the $Fe^{\cdots}$ is more rapidly reduced in the presence of excess of the reducing agent).

(3) Excess of iron causes a more stable coloration.

(4) Excess of alkali causes the coloration to be less *transient* (since $Fe^{\cdot\cdot}$ is increasingly unstable in alkaline solution).

Apparently the reduction potential of cysteïn does not develop with increasing alkalinity so rapidly as that of $Fe(OH)_2 \longrightarrow Fe(OH)_3$.

(5) With increasing alkalinity the *total duration* of the colour phenomena is less, since the cysteïn disappears more rapidly. (Cystine becomes more readily autoxidisable with increase of p_H.)

(6) It is found that the addition of *free cysteïn* to the coloured solution aids the (temporary) decolorisation. (Explanation as in (2).)

(7) It is a well known experimental fact that iron acts as a catalyst in accelerating the oxidation of cysteïn to cystine. This is readily explained on the basis of the above theory: the iron acts as a carrier; in the ferrous state it is a more efficient oxygen acceptor than the cysteïn, and oxidised it functions as an oxygen donator.

(8) The most convincing support to these views is afforded by the behaviour of copper (*vide infra*). It is found moreover that reversible colorations analogous with that given by iron could be obtained only by those metals which exist in more than one state of oxidation, and in which change of valency from the one form to the other can occur with tolerable ease.

With regard to the catalytic action of iron (see (7) above) mention should be made of the work of Mathews and Walker [1909] on the velocity of spontaneous oxidation of cysteïn in the presence of salt solutions. This paper was published prior to that of Arnold and I was unaware of the explanation therein advanced of the mechanism of the catalysis when the work described in the present paper was carried out.

"An explanation is given" by Mathews and Walker "of the action of different metals based on their solution pressure." These authors account for the catalytic action by assuming the momentary formation of an intermediate metallic compound

$$\text{"MeO + Cysteïn} = \text{Cysteïn . MeO.}$$
$$\text{Cysteïn . MeO} = \text{Cystin + Me + } H_2O\text{."}$$

It would seem that this view fails to account for the experimental observation that the coloured derivative is only instantly decomposed when excess of free cysteïn is present. The pure cupric cysteïn derivative can be isolated and shows no tendency for internal spontaneous scission such as is suggested.

The action of cysteïn upon the cupric and other coloured metallic derivatives, can leave little doubt that the mechanism of the reaction consists in the reduction of the oxidised metallic atom of the derivative by the action of excess of cysteïn.

The formation of various metallic derivatives of cysteïn.

Fe$^{\cdots}$. The behaviour of iron is explained above. The deep coloration obtained when (excess of) ferric chloride is added to cysteïn was first described by Andreasch, and then by Arnold. The latter author distinguished between the colour given by $FeCl_3$ and by "ammonia." With sufficiency of iron the colour remains until oxidation of cysteïn to cystine is complete, and cannot then be regenerated by aeration.

Fe$^{\cdot\cdot}$. Under ordinary conditions $Fe(OH)_2$ cannot be precipitated sufficiently free from $Fe(OH)_3$ to prevent the production of the violet ferric compound. Investigations have been started under anaerobic conditions.

Double salts (ferric alum, etc.) in which iron functions as $Fe^{\cdots}$ act like $FeCl_3$ or $Fe(OH)_3$. When iron functions in the complex anion as in K_4FeCy_6 or K_3FeCy_6 it is without action on the cysteïn anion.

Mn$^{\cdots}$ and **Mn$^{\cdot\cdot}$**. If a few drops of manganous sulphate be added to cysteïn (in HCl solution) and the liquid made alkaline with ammonia a fine *green* coloration (Mn-cysteïn cpd.) appears. It rapidly fades provided sufficient cysteïn be present. On blowing air through the faded solution the colour is regenerated. The general behaviour of manganese towards cysteïn resembles that of iron described at length above.

It appears probable that the green body is the $Mn^{\cdots}$ derivative and the decolorised solution contains $Mn^{\cdot\cdot}$. The addition of alkali to a manganous salt yields $Mn(OH)_2$, which however rapidly goes to $Mn(OH)_3$ or Mn_2O_3, H_2O

unless air be excluded. When cysteïn is also present the green colour is given in air, while in absence of air the solution remains colourless ($Mn^{\cdot\cdot}$). When oxidation of cysteïn has reached completion brown Mn_2O_3, H_2O remains. The addition of fresh cysteïn to this yields the green derivative and the sequence can then be repeated afresh.

Thunberg [1913] has found that in the presence of $MnCl_2$ the autoxidation of various thio-compounds is accelerated, a fact which is readily accounted for by a cycle such as the above, in which the salt functions as an oxygen carrier. According to Mathews and Walker [1909] however $MnCl_2$ has no effect on the velocity of oxidation of cysteïn.

Mn^{vi}. Dilute $KMnO_4$ added to cysteïn solution suffers decolorisation. On making alkaline with ammonia a *rose-red* colour appears, fading on standing and reappearing on aeration. The depth of colour is increased with increasing concentration of the two substances; excess of cysteïn producing more rapid fading on stoppage of the air current, coupled with a less readily regenerated colour on aeration, while increasing the amount of $KMnO_4$ gives a more stable coloration. Here again the behaviour is parallel with that of iron salts.

The rose colour may be due to sexvalent manganese, corresponding with MnO_3, and the colourless reduced solution may contain quadrivalent Mn— (corresponding with MnO_2) formed from the MnO_3 by the reducing action of excess of cysteïn.

The first stage would be the production of MnO_2

$$2KMnO_4 + 6H \cdot S{-}CH(NH_2){-}COOH = 2MnO_2 + 3(S{-}CH_2{-}CH(NH_2){-}COOH)_2 + 2KOH + 2H_2O.$$

In alkaline solution however Mn^{vi} is stable,

e.g.
$$Mn_2O_7 \xrightarrow[\text{alkali}]{} 2MnO_3 + O$$

$$3K_2Mn^{vi}O_4 + 2H_2O \underset{\text{in acid solution}}{\overset{\text{in alkaline solution}}{\rightleftharpoons}} 2KMn^{vii}O_4 + MnO_2 + 4KOH$$

hence we may have Mn^{vi} in the alkaline aerated cysteïn mixture. In the reduced solution MnO_2 is almost certainly present. The action of $KMnO_4$ is being further investigated.

$Cu^{\cdot\cdot}$ and **$Cu^{\cdot}$**. The existence of a copper derivative of cysteïn has been known for some time [Suter, 1895]. The addition of excess of a cupric salt to cysteïn in neutral solution produces a blue-black precipitate of a $Cu^{\cdot\cdot}$-cysteïn compound. The latter is almost completely insoluble in neutral or slightly acid reaction, and may advantageously be employed for the separation and estimation of cysteïn [Embden, 1901]. When the solution is somewhat acid it is better to employ moist freshly precipitated $Cu(OH)_2$ in place of the copper salt, following the procedure used by Hopkins [1921] in the separation of glutathione. The blue-black precipitate dissolves on addition of ammonia forming an intense dark brown (sepia) solution. It is reprecipitated on reducing the p_H to about 7 and dissolves to an almost colourless solution in excess of acid.

Interesting results have been obtained by the use of excess of cysteïn. The addition of cysteïn to the alkaline (brown) solution of the cysteïn-copper derivative causes rapid decolorisation. It will be shown that this is due to the reduction of the copper to the cuprous condition, and that the decolorised solution contains the cuprous-cysteïn derivative. If the decolorised solution be now shaken the dark brown colour reappears: the $Cu^{\cdot}$ has been re-oxidised by the air in the alkaline solution to $Cu^{\cdot\cdot}$. On standing, the solution fades once more; and these changes can be repeated so long as sufficient unoxidised cysteïn remains. By the prolonged action of oxygen or air on the brown solution the cysteïn is completely oxidised to cystine and a cuprammonium colour persists.

Copper is singular among the metals so far examined in that *both* its cysteïn salts (cuprous and cupric) are insoluble in neutral solution. As mentioned above an alkaline solution of the cuprous derivative is formed when the ammoniacal cupric—*i.e.* the brown—solution reduces spontaneously in the presence of excess of cysteïn. On neutralisation of the decolorised solution by the addition of dilute HCl the cuprous cysteïn compound is precipitated as a highly characteristic voluminous white precipitate. It may be obtained in a variety of ways. Add to a few cc. of a 5 % cysteïn solution one drop of $CuSO_4$aq. A blue-black precipitate of the cupric derivative first appears but is rapidly transformed into the white cuprous derivative. The $Cu^{\cdot\cdot}$ has become reduced to $Cu^{\cdot}$ by excess of cysteïn. The white precipitate is readily soluble in excess of acid or ammonia to a colourless solution. The alkaline solution readily darkens in presence of air owing to oxidation of $Cu^{\cdot}$. The blackened alkaline solution will of course again yield the cupric cysteïn derivative on neutralisation, while the air-free clear alkaline solution will give the white precipitate.

The cuprous derivative has also been obtained directly by the use of cuprous iodide. To a cysteïn solution a little well washed freshly precipitated cuprous iodide was added and the solution made alkaline with ammonia. An almost colourless solution resulted. This was neutralised and yielded the characteristic white precipitate. The strongly alkaline solution readily darkens in air—owing to oxidation of the cuprous ion—especially when the amount of copper is large. The colour can be seen extending downwards from the exposed surface of a large tube.

Co. The addition of a drop of cobalt acetate to cysteïn solution followed by ammonia resulted in the production of a dark yellow colour. No appreciable intensification resulted on prolonged passage of air through the mixture. The intensity of colour was increased by addition of further Co solution.

Ni. Cysteïn solution containing a little $NiSO_4$ assumed an orange colour on addition of ammonia. The colour deepened to a reddish brown (mahogany) appearance when some more cysteïn and nickel sulphate were added. The colour was retained so long as cysteïn remained in the solution.

Cr. On adding cysteïn solution to a weak, acidified solution of $K_2Cr_2O_7$

an intense chrome yellow colour appeared. On making alkaline with ammonia the usual greenish chromate colour was observed; it was not formed so readily however on the direct addition of ammoniacal $K_2Cr_2O_7$ to cysteïn. Potassium dichromate is peculiar in giving the coloration in *acid* solutions. Potassium chromate, which also contains the hexavalent chromium atom, behaves in the same way.

Sn^{iv}, $Sn^{\cdot\cdot}$. The formation of a tin derivative of cysteïn may be demonstrated in the following manner. Have two test tubes each containing 3 cc. of water, and to one add also about 30 mg. of cysteïn. Add a little $SnCl_4$ to both tubes. Now make both tubes alkaline by the addition of ammonia and then acid by means of sulphuric acid. The presence of a tin cysteïn derivative is shown by its ready solubility in acid and more especially in ammoniacal solution. The tube containing the cysteïn will be seen to become perfectly clear and colourless in presence of ammonia or larger excess of acid; while a bulky precipitate remains in the blank tube under the same circumstances.

It might be thought that the presence of $SnCl_4$ would favour oxidation of the cysteïn. There seems no doubt however that the production of the clear solution is due to unoxidised cysteïn, since its presence is shown by the nitroprusside reaction. Indeed the presence of $SnCl_4$ seems to inhibit the oxidation. In one experiment a merest trace of cysteïn was added to a bottle containing a large excess of $SnCl_4$ and water, and the mixture was made alkaline with ammonia. It still showed a nitroprusside reaction after the lapse of five days.

Tin has a particularly strong affinity for sulphur and no doubt it protects that atom in the cysteïn molecule from oxidation.

With $SnCl_2$ the results observed were in every respect identical with those described above for $SnCl_4$.

$As^{\cdot\cdot}$, $As^{\cdot\cdot\cdot}$. Arsenic appears to be too electro-negative to afford any ready evidence of functioning as the basic part of a cysteïn derivative. As_2S_2 and As_2S_3 were examined but without result.

Pb. The addition of $Pb(NO_3)_2$ to cysteïn solutions failed to yield a coloration.

$Hg^{\cdot\cdot}$. If insufficiency of "$HgSO_4$ reagent" is added to cysteïn the white precipitate first formed disappears. The same result can be obtained by addition of excess of cysteïn to the mercury precipitate. The phenomenon appears to be due to the reduction of the mercury from the bivalent to the univalent state, $Hg^{\cdot}$, which is inefficient as a precipitant. Analogous results may be obtained with other mercuric derivatives. If cysteïn be added in excess to a suspension (1) of *mercuric iodide*, or (2) of the *double ammonium compound* which is precipitated when ammonia is added to acidified mercuric iodide, the precipitate will be seen to dissolve in both cases.

When sufficient mercuric salt is added, however, precipitation occurs. Mercuric nitrate, chloride and acetate all yield insoluble compounds with cysteïn. These substances have no precipitant action on cystine.

Bi. Add cysteïn to an ammoniacal suspension of bismuth hydroxide (obtained from bismuth nitrate or subnitrate). The precipitate readily dissolves with the production of a yellow solution, indicative of a $Bi^{\cdot\cdot\cdot}$ cysteïn compound.

The action of BiO_4 and Bi_2O_5 remains to be investigated.

It will be seen that the observations recorded above open up considerable ground for further inquiry. A number of metals have still to be investigated. In view of the fact that considerable supplies of cysteïn will be required for continuation of the research and in view of the calls of other research work it has been thought advisable to give an account of the observations so far made.

I should like to express my sincere gratitude to Professor Hopkins for the help and encouragement he has given me and the interest he has shown in this work.

Summary.

(1) The so-called "ammonia" test for cysteïn is only effective in the presence of traces of a metallic compound.

(2) The following metals—viz. $Fe^{\cdot\cdot\cdot}$, $Mn^{\cdot\cdot\cdot}$, $Mn^{vi(c)}$, $Cu^{\cdot\cdot}$, $Hg^{\cdot\cdot}$—can form part of a system which is reversible so long as unoxidised cysteïn is present; the controlling factors being the relative concentrations of cysteïn, metal salt and atmospheric oxygen. In alkaline solution and in presence of oxygen, the reduced metal is equivalent to an oxygen acceptor, and in absence of oxygen the oxidised metal is equivalent to a donator of oxygen, the cysteïn forming the oxidisable body.

(3) The fading of alkaline solutions containing a metal *plus* cysteïn and the recoloration on aeration are explained on these grounds.

(4) Coloured metallic derivatives of cysteïn have been formed from $Fe^{\cdot\cdot\cdot}$, $Mn^{\cdot\cdot\cdot}$, $Mn^{vi(c)}$, $Cu^{\cdot\cdot}$, Co, Ni, Cr, Bi. $Cu^{\cdot}$ gives a characteristic white derivative insoluble in neutral solutions. Sn and Hg derivatives are also discussed.

(5) Cysteïn exhibits greater readiness to form metallic derivatives than is the case with cystine.

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LXXVIII. THE INFLUENCE OF FAT AND CARBOHYDRATE ON THE NITROGEN DISTRIBUTION IN THE URINE.

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(*Received September 22nd, 1922.*)

In previous papers it has been shown that the complete withdrawal of carbohydrate from the diet produces well marked changes in the metabolism. Landergren [1903] in his original paper showed clearly that (1) on an exclusively carbohydrate diet the output of total nitrogen steadily fell and (2) when the diet was changed to one composed exclusively of fat the output of total nitrogen rose. This finding I confirmed and extended [1909]. The series of experiments detailed in the present paper were intended to extend further our knowledge of the subject. They were all carried out previous to July 1914 on Mr R. Lang and it was intended to complete the series with the effects of such limited diets on the capacity of the individual to do muscular work, but unfortunately Mr Lang on his demobilisation was no longer available and so far no other subject, either willing or suitable to undergo the necessary privation, has been found.

Methods.

Previous to the ingestion of the various experimental diets the subject on each occasion attempted for two or three days to consume roughly the same diet so that the base line would be approximately the same. Unfortunately owing to circumstances over which the subject had no control this was not always possible. The life led by the subject was uniform throughout.

The oil used was the finest olive oil procurable. It was always emulsified before taking by shaking, after the addition of about 1 g. of potassium carbonate dissolved in water. In this form the oil was fairly readily consumed and during the three days of the duration of the experiment it gave rise to no untoward digestive disturbance or diarrhoea. As a matter of fact in one only of the three day experiments did a single movement of the bowels take place. Mr Lang found it impossible to carry on for more than three days on account of the nausea induced by the mere sight and smell of the emulsified oil. The addition of even 150 g. of glucose did not materially lessen this disgust although the subject always declared that a feeling of "tiredness" which was prominently associated with the ingestion of pure oil was distinctly reduced when sugar was added even in comparatively small amounts. The

sugar used was chemically pure anhydrous dextrose. The calorie values of the various diets were kept approximately constant; they varied between 3102 C. with pure oil and 2978 C. with oil plus 150 g. sugar. The analytical methods employed were: total nitrogen, Kjeldahl; ammonia, Folin; urea, Folin and urease; uric acid, Hopkins-Folin; creatinine and creatine, Folin.

Results.

Output of Nitrogen.

Diet	Day of experiment	In g. Total	Urea	Ammonia	Total creatinine	Uric acid	Undetermined	In percent. of T.N. Urea	Ammonia	Total creatinine	Uric acid	Undetermined
323 g. olive oil	1	10·00	7·31	0·32	0·50	0·094	1·76	73·1	3·3	5·0	0·94	17·6
	2	14·24	11·08	0·66	0·62	0·031	1·84	77·8	4·6	4·4	0·22	12·9
	3	[10·75]	7·75	1·07	0·63	0·033	1·27	72·1	9·9	5·8	0·31	11·8
323 g. olive oil	1	10·95	7·88	0·29	0·49	0·123	2·16	72·0	2·7	4·5	1·12	19·7
	2	14·35	11·05	0·56	0·52	0·059	2·17	77·0	3·9	3·6	0·41	15·1
	3	14·18	10·44	1·13	0·59	0·029	1·99	73·6	8·0	4·2	0·20	14·0
310 g. olive oil	1	9·41	6·94	0·21	0·58	0·136	1·53	73·7	2·2	6·2	1·44	16·2
30 g. dextrose	2	11·72	7·48	0·44	0·57	0·087	3·15	63·8	3·7	4·9	0·74	26·7
	3	10·23	6·60	0·58	0·60	0·066	2·38	64·5	5·7	5·9	0·64	23·2
297 g. olive oil	1	9·78	7·36	0·26	0·52	0·142	1·50	75·3	2·6	5·3	1·45	15·3
60 g. dextrose	2	9·31	7·07	0·31	0·49	0·119	1·32	75·9	3·4	5·3	1·28	14·2
	3	8·60	5·86	0·38	0·50	0·108	1·76	68·1	4·4	5·8	1·25	20·4
279 g. olive oil	1	7·97	5·38	0·26	0·48	0·133	1·73	67·5	3·2	5·9	1·67	21·7
100 g. dextrose	2	7·75	5·24	0·25	0·54	0·118	1·60	67·6	3·3	7·0	1·52	20·6
	3	7·12	4·79	0·18	0·54	0·145	1·47	67·3	2·5	7·6	2·03	20·6
257 g. olive oil	1	10·18	7·66	0·37	0·56	0·121	1·47	75·2	3·6	5·5	1·18	14·4
150 g. dextrose	2	9·95	7·28	0·24	0·56	0·152	1·68	73·2	2·4	5·6	1·52	16·8
	3	7·37	5·05	0·16	0·56	0·143	1·46	68·5	2·2	7·6	1·94	19·8

Total nitrogen.

The table clearly shows that with increasing amounts of carbohydrate there is a general tendency for the output of total nitrogen to decrease. On the pure oil, although in each instance there is a rise in the output of total nitrogen on the second day of the experiment, there is not the further rise noted on the third day of the experiment which both Landergren and I found in previous experiments. This may be due to the fact that in this series, in contradistinction to the earlier ones, olive oil emulsified by alkali was used in place of butter fat or cream. There is no doubt that the olive oil and alkali is much more readily tolerated by the intestine than either cream or butter. Instead of experiencing the acute diarrhoea which usually results from the ingestion of these products when taken alone, as above noted Mr Lang was constipated throughout the experiments.

Zeller [1914] has also carried out a series of observations, both on dog and man, on the effect of varying the fat and carbohydrate values of practically protein-free diets. It is to be regretted that his experimental periods on the fat-rich diets were so short, two days with the 75 % fat diet and only a single day with 100 % fat. Experiments of such very short duration do not permit

of the metabolism adjusting itself to the new conditions. The effect of the fat-rich diet had however the usual result, viz. a marked rise in the output of total nitrogen both in dog and man.

Urea.

The urea output resembles very closely that of total nitrogen. It is interesting to note that when the percentage outputs of urea and ammonia nitrogen are compared there is a very marked fall in the output of urea after the sugar, even with the smallest dose, although at the same time there is also a fall in the output of ammonia, whereas it might have been expected that with the decrease in the ammonia output there would have been a rise in the urea output. In the case of the experiments with oil alone there does seem to be a balance between the two outputs, a rise in the ammonia output being associated with a fall in the urea.

When the outputs of both ammonia and urea are taken together it may be definitely stated that when oil is given alone there is a definite and steady rise in the excretion of these nitrogenous materials, whereas in the experiments in which sugar is added there seems to be just as general a tendency for the united output to fall during the three days of the experiment.

Zeller in his series also found that the urea output followed very closely the curve for the output of total nitrogen. His percentage output of urea reached however a far lower level than any that I obtained. Thus when a pure carbohydrate diet was given immediately after a pure fat diet he found in one instance that urea only formed on the average 49·7 % of the total nitrogen and in the other it reached the very low level of 39·7 %.

Ammonia.

In each case with the pure oil diet a very definite steady rise both of the absolute and the percentage output of ammonia nitrogen occurs on the three days of the experiment. A slight rise is also noted on the third day when 30 and 60 g. of sugar are given although, even with these small amounts of sugar, there is a definite reduction in the total ammonia output. When the larger amounts of sugar are given there is a steady fall in the output of ammonia during the three days of the test.

Zeller too in his experiments found, like other observers, that the giving of fat-rich diets led to a marked rise in the output of ammonia. In all of his experiments, just as he found a much lower percentage output in the case of urea, he obtained much higher outputs of ammonia, both with the carbohydrate-rich and carbohydrate-poor diets, than those given in the present paper. His maximum percentage output of ammonia was 15·4 % with the 100 % fat diet.

Uric acid.

The variation in the excretion of uric acid as the result of the alterations in the composition of the diets is very striking and most interesting. As the

table shows it may generally be stated that when carbohydrate is completely removed from the diet there is a well marked reduction in the output and that this output gradually and steadily rises as the amount of glucose added is increased. It was shown in a previous paper [1909] that the output of uric acid varied with the carbohydrate intake; that the output was high when the subject was on a carbohydrate-rich diet and low when on a fat-rich, carbohydrate-poor diet. Graham and Poulton [1913] who studied the influence of carbohydrate and fat on the output of endogenous purine also found that a diet in which fat predominated was associated with a low output and a diet in which carbohydrate predominated with a high output of uric acid. Later Umeda [1915] working in my laboratory confirmed, both in the case of man and the dog, the variation in uric acid output as the result of alteration of the diet. He found in man that, although there was a definite fall in the output of total purines, the output of purine bases on a fat diet was higher than on a carbohydrate one. Graham and Poulton had also noted that when the carbohydrate content of the diet was reduced there was a tendency for the purine base output to rise. In the case of the dog, Umeda found that the allantoin output behaved like uric acid in its relation to the nature of the diet. Zeller also investigated the influence of carbohydrate and fat diets both on the output of uric acid and the purine bases. He too found that there was a reduction in the output of uric acid on a 100 % fat diet but there was little or no influence on the output of the purine bases. As already noted Zeller's experiments were of exceptionally short duration.

In an interesting and suggestive paper Ackroyd and Hopkins [1916] showed very definitely that (1) when both arginine and histidine were removed from the diet the amount of allantoin excreted in the urine of the rat was much decreased; (2) it was somewhat diminished when only one of these amino acids was present and (3) when both were restored to the diet the allantoin excretion returned to normal. Their conclusion that arginine and histidine probably constitute the most readily available raw material for the synthesis of the purine ring in the animal body would seem to be justified. Still, although these two amino acids may be considered to supply the raw material for the synthesis, this does not dismiss the probability that the presence of carbohydrate is necessary as in the experiments of Ackroyd and Hopkins the carbohydrate supply was abundant. Further, the well known experiment of Knoop and Windaus [1905] in which they demonstrated the formation of the iminazole ring when a solution of dextrose was acted upon by ammonia in the presence of zinc and sunlight, shows, at least, that a synthesis from very simple compounds is possible. If the rest of the diet played but a minor part in the synthesis of the purine it would have been expected that the output of purines would have risen when a general increase in the breakdown of the protein molecule took place, as is the case when oil alone is the food material supplied, *i.e.* when there would be in all probability an increased amount of amino acids including arginine and histidine free in the

tissues. Further the diminution of the output of uric acid on the fat diet is not due to delayed oxidation of other purine bodies because reference to the output of undetermined nitrogen, which would include purine bases, shows that the excretion on the fat diet is actually lower than when sugar is given. It is probable then that, although arginine and histidine may be regarded as the actual nitrogenous source of the purine, before the synthesis can take place carbohydrate in some form or other must be present.

Creatinine and Creatine.

Inasmuch as many of the estimations of these substances were made without the precautions suggested by Graham and Poulton the total creatinine output only is given. It may be stated that in two other experiments when every precaution was taken the presence of creatine in the urine was definitely shown. The work of Underhill and Baumann [1916] showed very conclusively that acidosis alone cannot account for the presence of creatine in the urine.

Undetermined nitrogen.

Although the absolute amount of undetermined nitrogen daily excreted does not vary very greatly in any of the experiments, yet, when calculated on a percentage basis, it is found that there is a very definite rise when carbohydrate is added to the diet. Zeller in one of his pure fat experiments found the highest percentage output whereas in the other the output was low, if not the lowest of the series. Zeller in addition to the substances referred to in this paper also determined amino and peptide nitrogen. The output of these substances rose when the pure fat diet was given.

Discussion.

The consideration of the question of isodynamic replacement and the inferences to be drawn from the consideration of experiments on complete replacement such as those described, irrespective of the question as to whether such experiments fall within the normal capacities of the tissues, open up very wide issues. The mere statement of the isodynamic law virtually upholds the thesis that the basis of nutrition is the exchange of energy and not the exchange of material or, at the very least, that *Kraftwechsel* predominates over *Stoffwechsel*. Rubner [1883] who enunciated the hypothesis that fat and carbohydrate are mutually replaceable in a diet in isodynamic amounts, although in a much more recent paper he definitely stated that it was impossible to replace completely any of the proximate principles, definitely selected the term isodynamic after the consideration of such less specific terms as *gleichwertig*.

The work of Rubner is generally stated to be substantiated by the work of Atwater and Benedict [1903]. The experiments of these workers do undoubtedly support it but the type of experiment they adopted could not be

expected to determine the ultimate degree of replacement, as only variations in a mixed diet on the human subject under approximately normal conditions were employed in contradistinction to Rubner's method of a rigorous administration of a single food stuff. It is undoubtedly true that within limits fat and carbohydrate may replace one another in the diet and it is obvious that in the average diet such an arrangement is automatically adopted. The statement of the case in the form of a general isodynamic law is simply untenable. At present the evidence is clear and convincing in support of the statement that it is impossible to replace carbohydrate completely by fat but so far the evidence available in support of the view that fat as such is a necessary constituent of a diet is scanty and unsatisfactory.

No one will of course seriously maintain that nutrition can ultimately be reduced merely to the satisfying of the energy demands: the calorie factor may be regarded as strictly secondary to the supply of material. We do not live on calories, yet all our general estimates of food requirements are quite properly for the most part made in terms of calories. Calorie value is simply a very convenient physical standard for the assessment of diets, but merely because such a standard has proved of great utilitarian value there is no real justification for placing this standard as the foundation stone of hypotheses framed to offer an explanation of cellular activity. Many writers are obsessed with the idea of the calorie, forgetting that the organism is certainly not a heat engine. It is perfectly true that calories are a measure of heat, but it must not be forgotten that we do not consume actual heat units but only potential heat-giving substances which can eventually be degraded to the form of heat and be measured as such. The thermal aspect of nutrition is unduly stressed, for, while heat may be a necessary product of tissue activity, it is, after all, a by-product.

The use of the term isodynamic in connection with problems of nutrition should be strictly limited. One can undoubtedly speak of isodynamic quantities of various substances but it does not follow that they are of equal, or indeed of any, value to the organism. When dealing with foodstuffs we ought to keep constantly in view that the material side is fully as important as the energy side. Therefore one ought not to stress so much the equality in energy as the equality in sparing or preventing tissue breakdown, the isoeconomic or, as I prefer to call it, the *isotamieutic* (Gk. tamieuo = to husband or to spare) value. Such a value is more physiological than isodynamic as it covers all phases of cellular activity. At present the data available do not suffice to permit of any adequate explanation of metabolic phenomena. Considerations such as those on which Carl Voit based his theory of metabolism, so actively rebutted by Pflüger, are not dismissed by the more modern hypothesis of Folin. Folin simply dealt with entirely superficial results and within these limitations the hypothesis is admirable. He did not attempt to elucidate the causal factors which lie beneath the phenomena which he correlated in his papers. The old question discussed so energetically by Voit and Pflüger as

to whether the newly ingested material becomes an integral part of the living molecule before utilisation is still unanswered.

Conclusions.

1. The output of total nitrogen, urea, and ammonia rises on a fat diet and falls on the addition of carbohydrate.

2. The output of uric acid is low on the fat diet and increases on the addition of carbohydrate.

3. The output of total creatinine is but little affected by the change of diet. Small amounts of creatine are excreted on a carbohydrate-free diet.

4. The output of undetermined nitrogen is greater on diets containing carbohydrate than on those from which carbohydrate is absent.

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LXXIX. ON THE INFLUENCE OF THE SPLEEN UPON RED BLOOD-CORPUSCLES. I.

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(*Received October 3rd, 1922.*)

Since the researches of Hunter [1892] on the destruction of red blood-corpuscles, an active rôle in this action has been ascribed to the spleen. The appearance of phagocytosis and haemolysis in the spleen had already been discovered by Koelliker [1847]. From this discovery resulted the researches of Virchow, Quincke and others on the transformation of blood pigments, in which for the rest the spleen was only considered as an organ of accumulation.

But it was Hunter who pointed out that the cause of the blood destruction partly lies in an action of the spleen itself. This view was based specially upon this author's researches on toluylene-diamine poisoning. As is known the action of this poison is much stronger in normal than in splenectomised animals, so that the opinion is justified that the spleen plays an interfering part in toluylene-diamine poisoning.

To determine the haemolytic power of the spleen the following four methods have been used:

1. Microscopical examination of spleen pulp in normal, experimentally modified and pathological conditions. This morphological method is not well adapted for learning the mechanism of the process observed.

2. Study of the consequences of splenectomy. We do not propose to discuss all the experiments made in this direction. The most generally accepted result of this operation is the increased resistance of the red blood-corpuscles of the animal against hypotonic salt solutions, a fact which has mainly been settled by the Dutch investigator Pel Jr. [1911].

3. Study of the action of splenic extracts. The results of this method, which appears to us as a very unreliable and inaccurate one, are moreover very contradictory.

4. Comparative examination of blood from the splenic vein and splenic artery. The latter may of course be replaced by arterial blood in general.

Destructive action of the spleen being admitted, it was obvious to investigate traces of this action in the blood of the vena lienalis. There are three principal qualities of this blood, which may be examined:

(*a*) the number of erythrocytes in a cubic millimetre;

(*b*) the amount of decomposition products of red cells, especially haemoglobin and bilirubin;

(*c*) the resistance of red blood-cells to haemolytic agents.

The number of erythrocytes in a cubic millimetre of the blood of the splenic vein, as compared with blood from the splenic artery, is not a very exact measure of the function of the spleen. In the first place the difference which might be expected would be so small as to be within the limits of technical error. If the lifetime of a red cell is estimated at 20 days (Quincke, Bénard; Rubner consider it to be at least 70–90 days, Dekhuysen still longer) and if one supposes that the total quantity of blood of the body passes through the spleen about thirty times a day [Burton-Opitz, 1912], it is clear that of each volume of blood which passes through the organ only $1/20 \times 1/30 = 1/600$ is destroyed. So one might expect to find numbers like 6,000,000 and 5,990,000 for splenic artery and vein. This difference is too small to be determined with sufficient accuracy. Larger differences might be found if this action of the spleen were intermittent, but there is no evidence that this is so.

Moreover the operation which is necessary for the puncture of the splenic vessels will cause a stagnation of the blood which has a great influence upon the number of erythrocytes.

Of late this method has been used again by Frey [1920]; his values for the difference between the blood from an artery of the abdominal wall and the splenic vein are so large (on an average 800,000 in a cubic millimeter) that in our opinion they must be due to some error in technique.

An increased quantity of bilirubin in the splenic vein, as compared with that in the blood from other vessels, might possibly be due to the formation of bilirubin in the spleen (Hymans van den Bergh and Snapper, Ernst and Szappanyos), it would not be conclusive proof of a haemolytic function of that organ.

The best method in our opinion is the investigation of the resistance of the red cells against hypotonic salt solutions, the so-called osmotic resistance. It is Eppinger [1920], who also stated that the osmotic resistance of the erythrocytes was decreased in the spleen, asserting that this fact is "eigentlich die einzig fassbare Tatsache die im Sinne einer zerstörenden Tätigkeit der Milz spricht, vorausgesetzt dass die Resistenzherabsetzung als ein einleitender Vorgang der Erythrozytenzerstörung aufgefasst werden kann."

French authors were the first to use the resistance determination in hypotonic salt solutions, originating from Hamburger [1904], to estimate the condition of the red blood-cells in several diseases. The value of this method however has been greatly increased by the modifications and extensions given by Brinkman [1922]. This author showed in the first place that the haemolysis in hypotonic NaCl solution is not only due to the difference of osmotic pressure, but also to the lyotropic effect of pure NaCl, the liquefaction of the cellular membrane-colloids by the Na-ions. Especially when the erythrocytes are washed several times with an isotonic NaCl solution, as is done by many authors, the cells are injured, as is shown by the diminished osmotic resistance (Snapper). This lyotropic effect may be abolished by the addition of Ca-ions to the solution in a very definite concentration. It would lead us too far to discuss the modified Ringer solution, which is used by Brinkman; we give here only the composition:

NaCl	...	...	0·7 %
$NaHCO_3$	...	...	0·2 %
KCl	...	...	0·01 %
$CaCl_2 6aq.$	...	...	0·02 %

$[H^{\cdot}] = 0{\cdot}45 \times 10^{-7}$.

Hypotonic solutions are obtained by diminishing only the concentration of NaCl.

If the red blood-cells are washed with this isotonic solution, the result is an increase of the osmotic resistance. This is due, as has been shown by Brinkman, to the removal of an autohaemolytic substance from the surface of the erythrocytes, a phosphatide, perhaps lecithin[1]. This same substance, if you make an emulsion of it in modified Ringer solution in an appropriate concentration, added to red blood-cells, may dissolve them.

In the surface of the blood-cells the haemolytic action of the phosphatide is balanced by the cholesterol, which is kept in solution by the first. The osmotic resistance has been shown to be dependent on the proportion cholesterol/phosphatide.

It must still be mentioned, that the modified Ringer liquid may be substituted by a mixture of primary and secondary phosphate, containing 16·30 g. Na_2HPO_4. aq. and 2·18 g. KH_2PO_4 per litre. The freezing-point of this liquid in which the erythrocytes have the same volume as in their plasma, is $-0{\cdot}46°$. Without any theoretical consideration, the equivalence of this solution with the modified Ringer solution has been proved empirically. Its advantages are the easiness of its preparation and its stability, combined with the physiological properties of the modified Ringer solution. It was necessary to mention this liquid, because we used it in our resistance-determinations. For further particulars reference must be made to the publications of Brinkman.

After this digression we will discuss briefly the results obtained by others in the determination of the resistance of red blood-cells from the splenic vein in comparison with that of erythrocytes from arterial blood.

Gabbi [1893] finds like Hammarsten an increased resistance in the splenic vein.

Pugliese and Luzatti [1900] observed however a lowered resistance of the red blood-cells leaving the spleen.

Chalier and Charlet [1913] once more found that both minimum and maximum resistance of the blood-cells from the splenic vein (*i.e.* respectively the resistance of the weakest and of the strongest erythrocytes in regard to haemolysis by hypotonic salt solution) were higher than those of erythrocytes from the splenic artery. From their observations they conclude that the spleen only lets the strongest erythrocytes pass and so exerts a haemolytic action upon the blood. This conclusion would be more justified, if only the minimum resistance had increased, the weakest erythrocytes entering the spleen having been destroyed. How is the increase of the maximum resistance, however, from this point of view to be explained, *i.e.* the fact that the most resistant blood-corpuscles which enter the spleen, leave the organ still more resistant?

The remaining investigators, Strisower and Goldschmidt [1914], Widal and Abrami [cited by Eppinger, 1920], Banti and Furno [cited by Eppinger, 1920], Polak Daniëls and Hannema [1916], Eppinger [1920], all found a *diminished* resistance of the erythrocytes leaving the spleen. We have already quoted Eppinger's opinion on the importance of this fact.

We also have tried to study the haemolytic function of the spleen by investigating its influence upon the resistance of the red blood-cells passing through the organ. For practical reasons we took our materials from sheep at the abattoir[2]. Immediately after the killing of the animal, some of the blood spurting out of the carotid artery was collected in a glass tube and defibrinated by shaking it with glass beads. Then as soon as possible the

[1] It may be obtained by evaporating the washing fluid and extracting the phosphatide from the dry residue, or, by direct extraction of the erythrocytes with chloroform, after they have been extracted with light petroleum, in which cholesterol and neutral fats dissolve.

[2] We were able to do so by the kindness of the authorities of the abattoir in Groningen.

spleen was removed, care being taken not to injure the capsule and the splenic vessels. The organ was then rapidly conveyed to the physiological laboratory. By introducing into the splenic artery a glass canula, which was connected with a reservoir, containing the modified Ringer solution, placed about 175 cm. above the working table, it was possible to obtain from the splenic vein the blood which had remained in the organ, diluted with the salt solution. The suspension of erythrocytes obtained in this manner was centrifuged immediately. These red corpuscles, which had been for some time in the spleen, longer than they are normally, but too short a time for autolysis to take place, seemed to us very well adapted for the investigation of an eventual resistance-decreasing power of the spleen. By the dilution of the blood from the spleen with modified Ringer solution, the resistance of the erythrocytes is practically not changed. The effect of washing red corpuscles with this solution, which we have already described, fails practically to be evident, if the total blood is "washed" with the liquid. To produce the same conditions, however, we have also diluted the arterial blood from the carotis and centrifuged it to get the corpuscles.

Both kinds of the corpuscles were then put in a series of tubes containing the phosphate mixture in geometrically decreasing concentrations, viz.:

9/10 isotonic (obtained by adding one vol. aq. dest. to 9 vols. of an isotonic solution).

$(9/10)^2$ isotonic (obtained by adding again one vol. of aq. dest. to 9 vols. of the 9/10 isotonic solution).

Etc.

Ten minutes after the addition of the erythrocytes, the latter were centrifuged and the degree of haemolysis read by Arrhenius' method. In 20 determinations we always reached the same result, that is to say, the blood from the spleen was *less resistant* than that of the carotis. Both the beginning and the complete haemolysis of the erythrocytes of the spleen occurred in a salt solution of higher concentration than those of the blood-corpuscles of the carotis. In a hypotonic salt solution of a definite concentration the degree of haemolysis of the blood from the spleen was always larger than that of erythrocytes from the carotis.

We do not think that *in vivo* the difference of the resistance of red blood-corpuscles from art. and vena lienalis will be as large as in our determinations, because in the latter the erythrocytes had remained for some time in the spleen. Eppinger observed *e.g.* that if the blood stagnated for 10 minutes in the spleen the resistance of erythrocytes from the vena lienalis was distinctly lower than before the stagnation. There is, however, evidence that *in vivo* also stagnation in the organ is possible.

Table I contains the determinations:

Table I.

		% haemolysis Salt concentrations						
No.	Kind of blood	Isotonic	0·9	$0·9^2$	$0·9^3$	$0·9^4$	$0·9^5$	$0·9^6$
1	Spleen	0	0	10	30	70	100	—
	Carotis	0	0	0	0	0	0	—
2	Spleen	0	0	0	20	50	90	—
	Carotis	0	0	0	0	0	0	—
3	Spleen	0	20	40	90	100	100	—
	Carotis	0	0	0	0	30	75	—
4	Spleen	0	5	10	50	70	100	—
	Carotis	0	0	0	0	5	10	—
5	Spleen	0	0	5	20	50	90	—
	Carotis	0	0	0	0	5	—	—
6	Spleen	0	0	0	50	90	95	100
	Carotis	—	—	—	0	5	60	90
7	Spleen	0	0	5	50	100	100	100
	Carotis	—	—	0	5	30	50	70
8	Spleen	—	—	10	30	50	100	100
	Carotis	—	—	0	10	50	80	100
9	Spleen	—	5	5	20	50	80	100
	Carotis	—	0	0	5	10	50	70
10	Spleen	—	0	5	10	40	60	90
	Carotis	—	0	0	0	5	30	90
11	Spleen	—	5	20	40	60	100	100
	Carotis	—	0	5	10	30	50	70
12	Spleen	—	0	0	10	50	70	100
	Carotis	—	0	0	0	10	50	70
13	Spleen	—	0	0	10	30	60	100
	Carotis	—	0	0	0	5	20	50
14	Spleen	—	0	5	10	40	80	100
	Carotis	—	0	0	5	30	50	70
15	Spleen	—	0	5	20	50	100	100
	Carotis	—	0	0	0	30	70	90
16	Spleen	—	0	5	10	30	70	100
	Carotis	—	0	0	0	30	70	90
17	Spleen	—	5	10	30	50	80	100
	Carotis	—	0	0	0	10	40	80
18	Spleen	—	5	15	30	80	100	100
	Carotis	—	0	0	10	30	70	100
19	Spleen	—	5	10	40	70	90	100
	Carotis	—	0	0	10	40	100	100
20	Spleen	—	5	20	50	100	100	100
	Carotis	—	0	0	10	60	100	100

If one calculates for every concentration the average degree of haemolysis one obtains the following table:

Table II.

Salt concentration	% haemolysis Carotis	Spleen
$0·9$ × isotonic	0	0
$0·9^2$ × ,,	0	5
$0·9^3$ × ,,	0	30
$0·9^4$ × ,,	20	60
$0·9^5$ × ,,	50	85
$0·9^6$ × ,,	75	100
$0·9^7$ × ,,	100	100

One may consider this as an accidental case. Fig. 1 shows the "resistance-curves" of this case (the left one of the corpuscles of the spleen, the other of the carotis corpuscles):

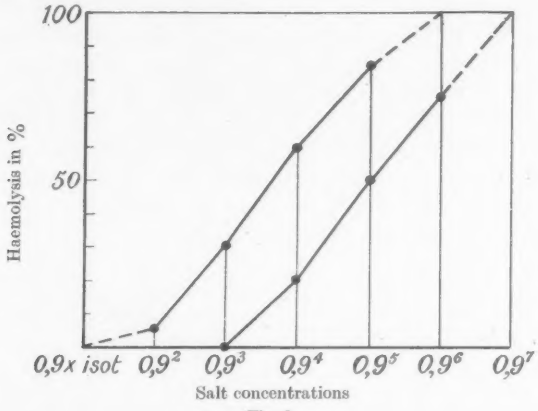

Fig. 1.

To deduce from the decreased osmotic resistance of the erythrocytes leaving the spleen conclusions as to the haemolytic function of the organ, it is necessary to investigate how far the phenomenon is specific. Of course it is not necessary that only the erythrocytes from the vena lienalis should have a decreased resistance; it might be also the case with blood-corpuscles leaving the liver and perhaps other organs in so far as these are concerned with blood destruction. We have contented ourselves for the present with showing that an organ like the kidney to which no haemolytic function is assigned fails to show a resistance-diminishing power. Table III shows our determinations:

Table III.

		% haemolysis Salt concentrations					
No.	Kind of blood	0·9 isotonic	$0·9^2$	$0·9^3$	$0·9^4$	$0·9^5$	$0·9^6$
1	Kidney	0	0	5	50	75	100
	Carotis	0	0	5	60	75	100
2	Kidney	0	5	10	50	80	100
	Carotis	0	5	10	30	50	70
3	Kidney	0	0	10	50	80	100
	Carotis	0	0	10	30	70	100

We have already mentioned the opinion of Chalier and Charlet and of Gabbi, that the spleen would exert a destroying action upon the weakest blood-corpuscles, so that the result is an increased resistance of the erythrocytes in the splenic vein, as was found by these authors, and we have observed that it is difficult to explain the increase of the maximum resistance from this point of view. From our researches, however, it would follow that the spleen exerts a haemolytic influence upon all blood-corpuscles, passing through the

organ, as is proved by the diminished minimum and maximum resistance of erythrocytes from the vena lienalis. The parallel course of the curves in Fig. 1 gives a clear image of this fact. If one makes *e.g.* the assumption that the spleen produces an internal secretion, which causes an alteration of all erythrocytes passing through the organ—and perhaps also has some influence upon the bone marrow—the mode of action of the spleen becomes more intelligible. We also point to the fact that after splenectomy a general increase of the resistance of the blood-corpuscles has been found, *i.e.* an increase of the minimum and maximum resistance whilst the "breadth of resistance" (the difference in concentration between the solutions in which beginning and complete haemolysis is observed) remains the same. Pel gives *e.g.* the following table as the average of a large number of experiments:

Table III

	Normal dog	Splenect. dog
	%	%
Average concentration of NaCl, in which beginning haemolysis is observed	0·42	0·35
Average concentration of NaCl, in which just complete haemolysis is observed	0·30	0·23
Breadth of resistance	0·12	0·12

This phenomenon would also be explained in the most simple way by the assumption that after removal of the spleen the production of an internal secretion ceases, that has normally a harmful action upon the erythrocytes, which is measured by the diminished resistance.

We have tried to ascertain more particulars about the resistance-diminishing power of the spleen. Assuming the action of an internal secretion, it was obvious that in the first place the surface layer of the erythrocytes would be altered by such an action. From the researches of Brinkman and Miss van Dam [1921] we know however the importance of the proportion cholesterol/phosphatide in the surface layer for the osmotic resistance. A decrease of this proportion means a diminution in the osmotic resistance. To test the assumption that only or principally the surface layer of the erythrocytes is altered by the action of the spleen it is only necessary to investigate the resistance of the blood-corpuscles of the vena lienalis, when this surface layer has been removed by washing the erythrocytes with modified Ringer solution. We have made these determinations by washing the blood-corpuscles obtained in the manner described on p. 757 and also the erythrocytes from the art. carotis three times with the modified Ringer solution and then determining the resistance in the same way as described above. In all our determinations we found a considerable difference between the two kinds of corpuscles, before washing, which disappeared however almost entirely after this process. Table IV contains the values of these determinations:

Table IV.

No.[1]	Kind of blood. Three times washed with modified Ringer	% haemolysis. Salt concentrations: Isotonic	0·9	$0·9^2$	$0·9^3$	$0·9^4$	$0·9^5$	$0·9^6$
1	Spleen	0	0	0	0	5	20	—
	Carotis	0	0	0	0	0	5	—
2	Spleen	0	0	0	0	20	50	—
	Carotis	0	0	0	0	0	0	5
3	Spleen	0	0	0	0	5	20	—
	Carotis	0	0	0	0	0	5	—
4	Spleen	—	0	5	10	30	50	100
	Carotis	—	0	0	10	30	50	100
5	Spleen	—	0	0	0	5	10	40
	Carotis	—	0	0	0	5	10	40
6	Spleen	—	0	0	10	40	80	100
	Carotis	—	0	0	10	40	80	100
7	Spleen	—	0	0	20	50	70	100
	Carotis	—	0	5	20	30	70	100
8	Spleen	—	0	0	10	30	60	90
	Carotis	—	0	0	20	30	50	90
9	Spleen	—	0	0	0	10	40	100
	Carotis	—	0	0	0	10	30	70
10	Spleen	—	0	0	10	40	80	100
	Carotis	—	0	0	20	50	80	100

Table V contains the average values which are represented also in Fig. 2.

Table V.

Salt concentration	% haemolysis: Washed spleen Corpuscles	Washed carotis Corpuscles
0·9 × isotonic	0	0
$0·9^2$ × ,,	0	0
$0·9^3$ × ,,	5	5
$0·9^4$ × ,,	20	15
$0·9^5$ × ,,	45	35
$0·9^6$ × ,,	90	85

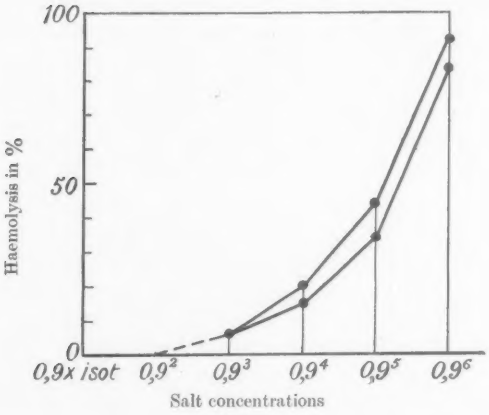

Fig. 2.

[1] The resistance of the unwashed corpuscles of these cases may be seen respectively in Nos. 1, 2, 3, 4, 11, 13, 14, 15, 16, 17, 18 of Table I.

The resistance of the arterial erythrocytes does not increase in the same degree as that of the blood-corpuscles of the spleen, so that after the washing the difference between the two kinds of blood-corpuscles has been reduced considerably, as is shown by Table V and Fig. 2.

There remains a small difference; this is perhaps partly due to the defibrinating of the blood from the art. carotis (the resistance is increased hereby somewhat); moreover arterial erythrocytes are always somewhat more resistant than blood-corpuscles from venous blood (Hamburger). The same difference exists between the resistance of erythrocytes from the vena renalis and art. carotis.

From our experiments we draw the conclusion that the influence exerted by the spleen upon the red blood-cells is confined to the adsorbed surface layer of the latter, *i.e.* to the layer which may be removed by washing with a modified Ringer solution. As we have not found any conception in the literature about the mode of alteration of the erythrocytes themselves in the spleen, except the following of Jacobsthal [1921], it seemed worth while to quote it. "Ich habe mir die Vorstellung gebildet, dass die Abnahme der Blutkörperchenresistenz beim haemolytischen Ikterus sich so entwickelt, dass die die Milz passierenden Blutscheiben jedesmal nicht haemolysiert, sondern *an ihrer Lipoidhülle sozusagen nur angenagt werden.*"

The three following alterations in the surface layer of the red blood-corpuscles in the spleen are possible:

1. Amount of phosphatide the same; amount of cholesterol decreased.
2. Amount of phosphatide increased; amount of cholesterol the same.
3. Amount of phosphatide and cholesterol both increased, the former however in the highest degree.

In all these cases the proportion cholesterol/phosphatide would diminish and this means a decrease of osmotic resistance. It seems that of these three possibilities the third one holds true. It has appeared to us that the erythrocytes leaving the spleen contain more cholesterol than the erythrocytes from the art. carotis[1]. It is therefore necessary that the phosphatide should undergo a considerable augmentation. We want to point out here already that this increase may be as well of a qualitative as of a quantitative nature. The specific influence of the phosphatide and cholesterol of the surface of the erythrocyte upon its resistance is undoubtedly due to the physico-chemical properties of these substances. If one makes an emulsion of lecithin *ex ovo* in an isotonic modified Ringer solution, this emulsion has a low surface tension and a great haemolytic power. If, however, cholesterol is added in increasing small amounts, the surface tension and the viscosity of this emulsion rise gradually and at the same time the haemolytic power decreases. The haemolytic power of an emulsion of lecithin is dependent on the *quantity* of the substance per cc. of the solvent, but apparently small *qualitative* alterations of the lecithin also have an enormous influence upon its haemolytic power.

[1] Publication of these investigations will follow.

This has been shown by Delezenne and Fourneau [1914], who withdrew oleïc acid from lecithin by means of cobra venom and in this way obtained a substance, called by them "désoléolécithine" or "lysocythine," which had a very strong haemolytic power. It is possible that *in vivo* also analogous chemical changes play a part in haemolytic processes. Perhaps it will be possible to settle the nature of the actual quantitative or qualitative changes in the phosphatides in the erythrocytes in the spleen and elsewhere by further researches.

To judge the value of our opinions, developed after investigations on the sheep's spleen, for normal and pathological physiology (Eppinger's Hypersplenie) of the human spleen, it will be necessary to repeat the determinations of the resistance described by us in man. This is possible where splenectomy is performed. Till now only one case has been investigated in this respect. For these figures we are indebted to Dr H. H. de Zoo de Jong, curator of the clinic of Prof. L. Polak Daniëls, University Hospital, Groningen. Splenectomy was performed in the case of a haemolytic anaemia. Some blood was taken from the vena lienalis and the red blood cells compared with erythrocytes from the peripheral blood in regard to their osmotic resistance before and after washing with a modified Ringer solution. Table VI contains the determinations:

Table VI.

	% haemolysis			
	Before washing		After washing	
Salt concentration	Peripheral	Spleen	Peripheral	Spleen
$\frac{1}{2}$ isotonic	20	30	0	0
$0{\cdot}9 \times$ "	35	65	40	40
$0{\cdot}9^2 \times$ "	70	80	70	70
$0{\cdot}9^3 \times$ "	80	85	80	80
$0{\cdot}9^4 \times$ "	95	95	90	95
$0{\cdot}9^5 \times$ "	100	100	100	100

The difference between peripheral blood and blood from the vena lienalis would perhaps have been more evident if the resistance had also been determined in solutions stronger than $\frac{1}{2} \times$ isotonic. Then the beginning of haemolysis would also have been determined.

For the rest it is clear that in this case the investigations on the sheep's spleen are wholly confirmed. It will, however, be necessary to repeat these determinations in regard to man if possible.

Conclusions.

The spleen has the power of *diminishing* the *osmotic* resistance of the erythrocytes. These are prepared hereby for haemolysis, which partially takes place in the organ itself.

Erythrocytes from the vena lienalis, which have been washed with an equilibrated salt solution, *do not show* this decreased osmotic resistance. This fact proves that the point of attack of the haemolytic power of the spleen lies in the removable surface layer of the erythrocytes. The alteration in this layer must consist in a decrease of the proportion cholesterol/phosphatide.

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LXXX. THE ENZYMES OF THE LATEX OF THE INDIAN POPPY (*PAPAVER SOMNIFERUM*).

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(*Received October 3rd, 1922.*)

This paper must be considered in the nature of a preliminary note. The work done has simply been qualitative and pressure of other work has prevented a more detailed treatment hitherto. In view of certain interesting results obtained and as it is possible the work may have to be discontinued it is deemed worth while to put it on record.

When poppy capsules are lanced for opium[1] the latex which immediately exudes varies considerably in colour from capsules in the same field and from the same pure race of poppy and even at times from capsules on the same plants. It may be pure white, smoky grey, light pink or deep pink in colour. It rapidly darkens to some shade of brown, either light chestnut or deep mahogany and at times it is almost black. When this opium is dried at air temperature and powdered the resulting powder may vary from a pale straw colour to almost black.

Moreover in a field sown with a single pure race of poppy, samples of opium collected on the same day at the same time under apparently identical conditions, on being subsequently air dried and powdered vary considerably in colour, *i.e.* from pale straw colour to almost black.

It would appear probable that the change is due to oxidation and one would expect to find powerful oxidising enzymes in the latex.

That the change is due to oxidation seems proved by the following experiments.

A. Some of the fresh latex diluted slightly with water to make it more liquid was drawn into the bulb of a Lunge nitrometer over mercury. The tap was turned off, thus leaving some of the latex exposed in the cup of the nitrometer. The remainder of the latex was effectually sealed up by the mercury out of contact with air. The latex exposed to air rapidly darkened. That sealed up after three months still shows no sign of darkening.

B. Portions of fresh latex were placed in small distillation flasks which were exhausted with a Geryk pump and sealed off. After three months these samples still show no sign of darkening whereas latex in similarly sealed flasks which were afterwards cracked rapidly darkened.

[1] For details of method of lancing see Annett [1921].

Latex kept in closed vessels over alkaline pyrogallol darkens because the complete absorption of oxygen by this reagent is a slow process unless the vessel is vigorously shaken and this is not feasible in the presence of the latex.

These preliminary experiments would appear to show that the darkening of the poppy latex is due to an oxidation process. It therefore appeared of interest to examine the latex for the presence of oxidising enzymes.

(1) Some fresh latex was shaken with water and divided into portions (*a*) and (*b*). The flask containing (*a*) was placed in boiling water for two minutes. (*a*) and (*b*) were then both filtered. Next day the filtrate from (*b*) was much darker than the filtrate from (*a*), thus indicating that the boiling had destroyed the oxidising enzyme.

(2) Latex mixed with water and fresh guaiacum tincture gave no blueing but when the test was repeated in presence of H_2O_2 a strong blue colour was immediately produced. It would therefore appear that a peroxidase is present in the latex. Spence [1908] working with rubber latex obtained a similar result but on dialysis with water for 24 hours he obtained both oxidase and peroxidase.

(3) Some fresh latex was immediately tested for oxidising enzymes with the following results:

(*a*) Latex and water + guaiacum tincture gave an immediate green colour but when the test was repeated in presence of H_2O_2 no reaction was obtained.

(*b*) A similar test with benzidine gave a deep colour immediately in absence of H_2O_2, but when the test was repeated in presence of H_2O_2 no reaction was obtained.

(*c*) A similar test with tyrosine suspension in absence of H_2O_2 gave darkening within 15 minutes and next day the liquid was very dark. On repeating the test in presence of H_2O_2 no darkening was obtained.

(*d*) Pyrogallol gave similar results, *i.e.* oxidation in absence of H_2O_2 but no reaction in its presence.

This result is directly opposed to that obtained under (2) above. The latex used under (3) was, however, used within 20 minutes of the lancing of the capsules while that used under (2) was probably two hours old. Some of the same latex used for (*a*), (*b*), (*c*) and (*d*) above tested about an hour later gave similar results except in the case of benzidine with which reagent a strong reaction was obtained both with and without H_2O_2.

In these cases controls were done with boiled liquids and all gave negative results.

This would indicate that the relative freshness of the two lots of latex used had nothing to do with the different results under (2) and (3).

(4) 10–15 g. of latex, collected for experiments in (3) above, were rubbed up in a mortar with distilled water and poured into a cleaned goat's stomach. This was allowed to diffuse in a vessel through which a slow stream of tap water was passed. The tap water was passed through thymol before reaching the diffusing vessel. Next day the walls of the goat's stomach were quite

black, indicating the presence of tyrosinase in the latex. 100 cc. of the dialysed liquor, the total volume of which was about 300 cc., were removed for enzyme tests. It was very faintly acid in reaction to litmus paper. 40 cc. of the liquid were boiled in a water-bath for five minutes. The table sets out the results of experiments then performed with the boiled and unboiled dialysed liquid. 3 cc. of the liquid were taken for the test in each case: "−" indicates no reaction, "+" a reaction, and "++" a very powerful reaction. Where H_2O_2 was used one drop of the 10 volume reagent was employed.

	Unboiled dialysed liquid		Boiled dialysed liquid	
Reagent	No H_2O_2	With H_2O_2	No H_2O_2	With H_2O_2
Guaiacum	++	−	−	−
Benzidine	++	−	−	−
Pyrogallol	+ ++ next day	−	−	−
Tyrosine	+ ++ next day	−	−	−

After another two days more dialysed liquid was removed from the goat's stomach. It was then quite neutral to litmus. The above tests were repeated with exactly similar results except that the unboiled liquid gave a very faint reaction with benzidine in presence of H_2O_2.

Another portion of the dialysed liquid was filtered and tested as above. Almost identical results were obtained. That is to say reactions were obtained with guaiacum, benzidine and tyrosine in absence of H_2O_2. In the case of guaiacum and tyrosine no such reaction was obtained in presence of H_2O_2, but in the case of benzidine a very faint reaction was obtained in the presence of H_2O_2.

(5) Some latex was collected and tested within 10 minutes of lancing with guaiacum and a deep green colour was immediately obtained. The test when repeated in presence of H_2O_2 gave an even more intense reaction. This is again a contradictory result.

The rest of this latex was shaken with water and divided into two portions *A* and *B*. *A* was rendered faintly alkaline with $NaHCO_3$ and one-half of it, *A'*, was boiled in a water-bath for five minutes. One-half of *B* was boiled similarly and is called *B'*. *A*, *A'*, *B* and *B'* were then tested with guaiacum, benzidine, and tyrosine with and without addition of H_2O_2.

The boiled solutions gave no reaction in any case. The reaction with guaiacum in case of both *A* and *B* solutions was very doubtful probably owing to the latex having been too largely diluted.

With benzidine both in the case of *A* and *B* a very strong reaction was obtained in absence of H_2O_2, the reaction being distinctly stronger in the alkaline solution. In the presence of H_2O_2 a faint but positive reaction was obtained with both solutions *A* and *B* but the reaction was much weaker than in absence of H_2O_2.

With tyrosine a practically similar result was observed namely a strong reaction in absence of H_2O_2 in both solutions *A* and *B*, but it was not more

marked in the alkaline solution. In presence of H_2O_2 a very faint but positive reaction was obtained in the case of both *A* and *B* solutions.

(6) Some of the latex collected for tests under section (3) was immediately after collection rubbed up with water and poured into a tall cylinder. Toluene was poured on the surface and the cylinder allowed to stand undisturbed. The insoluble portions of the latex settled out at the bottom of the cylinder. It will be remembered that when tested under section (3) a strong reaction was given with both guaiacum and benzidine in absence of H_2O_2 but there was no reaction in presence of H_2O_2.

After the cylinder had stood for eight days some of the liquid was drawn up from the bottom with a pipette. It contained pieces of the insoluble matter of the latex. This liquid reacted with guaiacum both in presence and absence of H_2O_2 the reaction being slightly stronger in the latter case. It reacted with benzidine in absence of H_2O_2 but not when H_2O_2 was present.

(7) It appeared of interest to test old powdered opium for oxidising enzymes.

Some opium powder two years old was rubbed up with water and dialysed. The dialysed liquid was then tested with guaiacum and benzidine in presence and absence of H_2O_2. Guaiacum gave no reaction in either case; benzidine reacted strongly in absence of H_2O_2 but in presence of H_2O_2 there was no reaction.

Tests for other enzymes in the latex.

We have been unable to find any reference to work on the enzyme content of poppy latex, with the exception of a statement that it contains only a small amount of protease [Czapek].

Fifty capsules were lanced and the latex which exuded was immediately scraped off. It was macerated with sand and 100 cc. water. After straining through coarse cloth the filtrate was divided into two portions *A* and *B*. The latter was boiled. The usual tests for enzymes were then carried out on the following substances: 1 % starch solution, 1 % cane sugar solution, 1 % α-methyl glucoside solution, 1 % urea solution and white flour extract (10 %). The result of these tests indicated the complete absence of amylase, invertase, maltase and urease. The test for protease indicated the possible presence of protease in small quantity as a very faint positive result was obtained.

Emulsin was tested for with amygdalin as in Armstrong and Horton's test, and a negative result obtained.

There was one interesting observation we made in all these tests, namely that the liquids in the tests with unboiled latex all darkened distinctly with reference to the control experiments carried out with boiled latex. This is another indication of the presence of oxidising enzymes.

Conclusions.

1. The darkening of the latex of the Indian Opium Poppy is a process of oxidation.

2. The latex has a powerful oxidising action on guaiacum tincture, pyrogallol, benzidine and tyrosine in the absence of H_2O_2. In almost all the experiments tried it was found that H_2O_2 inhibited these reactions.

Chaudat and Staub [1907] found that hydrogen peroxide retarded the action of tyrosinase.

Bach [1906] and also von Fürth and Jerusalem [1907] have found that though hydrogen peroxide in certain amounts retards the action of tyrosinase, yet minute quantities accelerate the action of the enzyme.

3. The dialysed latex both before and after filtration also oxidises guaiacum tincture, pyrogallol, benzidine and tyrosine in absence of H_2O_2 but the reaction is inhibited in the presence of this reagent.

4. The actions on benzidine and tyrosine were particularly powerful. In the case of tyrosine the darkening always appeared as a surface film on the liquid and gradually diffused downwards.

5. Opium powder stored for three years has been shown to possess an oxidising enzyme which acts on benzidine.

The author has recently shown that dry opium powder loses a considerable proportion of its morphine on storage. This may be due to the action of oxidising enzymes.

6. The following enzymes were tested for in the latex and not found, viz. amylase, invertase, maltase, emulsin and urease. There were indications of a weak proteolytic activity.

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LXXXI. THE BLOOD OF EQUINES.

By CHRISTIAN PETRUS NESER.

Preliminary Communication[1] *from the Division of Veterinary Education and Research, Onderstepoort, Union of South Africa.*

(*Received October 3rd, 1922.*)

Technique. Without offering details of technique it may be briefly stated that blood, drawn from the jugular vein except where otherwise mentioned, was collected in 10 cc. bottles containing a measured adequate amount of sodium citrate solution. From this were determined, (*a*) percentage volume of erythrocytes by centrifuging in specially prepared uniform tubes of 2 cc. capacity, blown out slightly at the sealed end to facilitate rapid sedimentation; (*b*) corpuscle count after diluting 1 in 200 with Hayem's fluid for erythrocytes, and 1 in 10 with 0·5 % acetic acid for leucocytes, the Bürker chamber being used in both cases; (*c*) haemoglobin content by Sahli's method.

Smears were made by Craandyk's [1918] method and also by a modification of Ehrlich's method which is so simple and effective as to merit brief description. A small drop of blood is quickly transferred to a slide by means of a suitable platinum loop, and a cover slip, as broad as the slide, immediately lowered over it. The droplet at once spreads as a thin circular layer, and on drawing the slip lengthwise over the slide all the blood is left upon the latter as a thin film in which the distribution of leucocytes is remarkably uniform, and eminently suitable for differential count after Giemsa staining in the usual way.

Limits of error. From numerous observations expressly designed to determine the degree of accuracy of methods in conventional use, it was concluded that (*a*) the centrifuge gives very accurate and consistent data, (*b*) red counts are liable to an error of up to 10 % even with the improved Bürker chamber, (*c*) white counts are less liable to error, (*d*) the Sahli reading cannot be relied upon to within five scale units, (*e*) smears made by the described modification of Ehrlich's method show a very regular distribution of leucocytes.

The red corpuscles.

Influence of work. Comparative study of the blood of different horses showed a remarkable difference in percentage volume of erythrocytes from individual to individual, but a fairly constant figure for any given individual over short intervals of time. The outstanding difference between the blood of different horses was at first very puzzling. Since all were fed alike, diet

[1] The full paper, giving detailed protocols, will appear in the forthcoming "Report of the Director of Veterinary Education and Research" (P.O. Box 593, Pretoria).

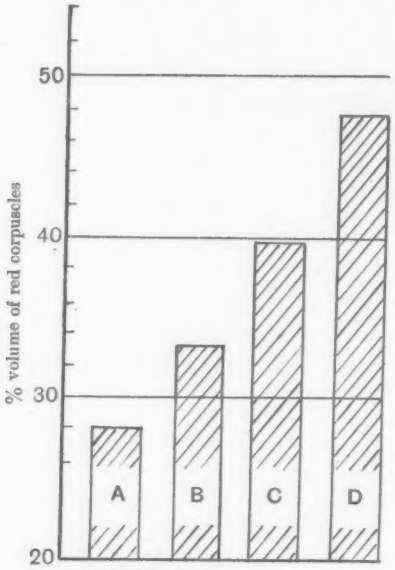
was not a factor. Age was excluded by statistical comparison of data. That sex played no part was evidenced by similar variation in mares and geldings. Since all the animals under observation had been purchased for the horse-sickness experiments of the institution, they were a mixed lot whose histories were generally unknown, and no mere inspection sufficed to explain the observed variations.

The clue, however, was given by two animals (laboratory Nos. 11144 and 11775) one of which was permanently stabled and showed a percentage volume of 23, while the other was used as a saddle horse and showed a figure of approximately 40. This remarkable difference at once suggested that *work* was the deciding factor in determining the percentage volume of erythrocytes in horse blood. Following up this clue, horses were grouped according to the work performed, as "fast working," "permanently stabled," and "other horses." It was at once noted, *without exception*, that the blood of fast-working horses showed a high percentage volume of erythrocytes, or high red count, while in horses stabled for six months or more the figures were correspondingly low. With the third group the data were variable, but it is probable that if the previous histories of these miscellaneous animals had been known, many of them would have been classified in one of the other groups. At a later date it was found possible to procure the blood of a few race-horses in training, and their high volume of red corpuscles bore out the earlier conclusions in most striking fashion. Graph I represents a summary of the data from over 200 animals from all sources, Graph II the data from three race-horses in various stages of training, while Graph III illustrates the change actually occurring during hard training of three young race-horses.

These data leave no doubt that hard fast work brings about a marked increase in the percentage volume of red blood corpuscles in the horse. The contrast shown in Graph II, for three race-horses from the same stable, is particularly striking, the figure for the fully trained animal showing that 52 %, or more than half the total volume of blood, may be made up of erythrocytes. This corresponds to a count of approximately 12 million as against an average count of about 8 million for the ordinary slow working horse, and less than 6 million for permanently stabled laboratory horses.

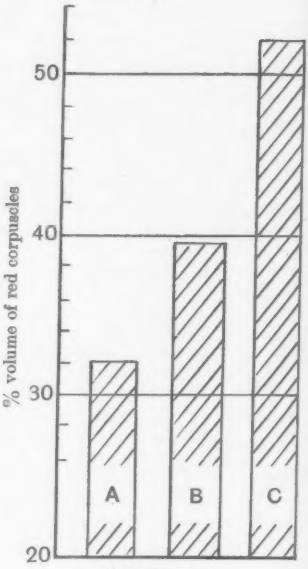
These already conclusive observations are further clinched by reference to Graph III, which shows a steady increase in numbers of red corpuscles during the actual course of hard training. In five weeks the count increased from less than 7 million to over 9 million in one case, and from 6·3 million to 8·8 million in a second case. In the third case training was interrupted owing to injury about the third week, the rapidly rising count then remaining stationary. Of special interest is the further fact that although the high count follows so rapidly in the wake of hard training, the reverse effect, or diminution of red blood corpuscles with rest, is a slow process. Thus in two young horses a decrease of two millions occurred only after three months, while in several old animals the decrease after five months was hardly noticeable at all.

Graph I.



A, Stabled horses.
B, Medium horses.
C, Fast working horses.
D, Race-horses in full training.

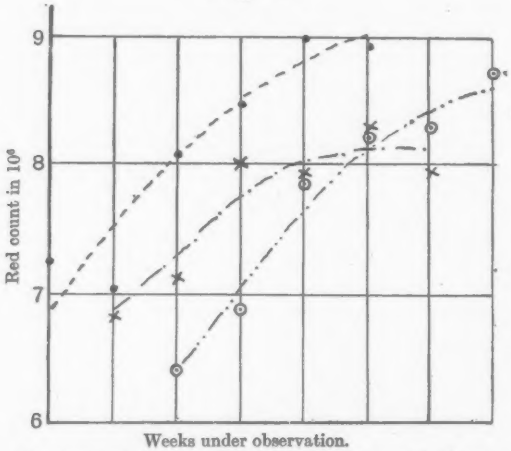
Graph II.



Race-horses.
A, Untrained.
B, Partly trained.
C, Fully trained.

Showing the influence of hard fast work upon the % volume of red corpuscles.

Graph III.



Showing the influence of time upon the red count in three young race-horses, during hard training.

Relationship between volume, count, and measurement of erythrocytes. In numerous cases, the percentage volume as determined by the centrifuge, the actual count in the Bürker chamber, and the diameter of the cells as measured in smears, were compared for the same horses.

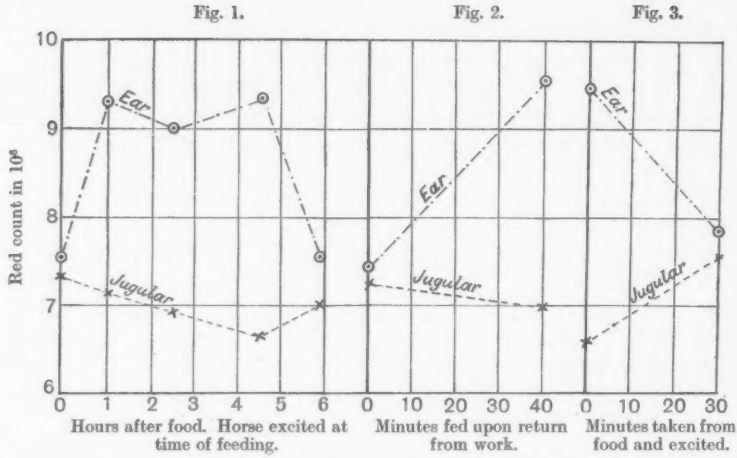
The *diameter* of individual corpuscles varied between 4μ and 8μ: but, with one exception, the average of 200 cells remained fairly constant at $5{\cdot}5\mu$. In respect, therefore, to average size of erythrocytes, different horses are very uniform.

The *ratio* between percentage volume and numerical count of red blood corpuscles varied between $4k$ and $4{\cdot}7k$, with an average of $4{\cdot}35k$—k being a constant dependent upon the units of measurement used. From the relatively constant average ratio, the observed liability of the red count to an error of 10 %, and the observed constancy of centrifugal readings, it may be concluded that the variations observed are mainly due to errors in counting and not to real variations in the average size of the corpuscles. These considerations led to the conclusion that a count calculated from percentage volume on an assumed ratio of $4{\cdot}35k$ is even more correct than an actual count; at least for the blood of healthy horses. It is quite certain that the percentage volume and the red count give the same information, and that the observed increase of the former with work is due to a real increase in the number of normal red cells per unit volume of blood; normal also in respect to haemoglobin content as determined by the Sahli method. This increase in erythrocytes is probably absolute, and a genuine response of the blood-forming tissues to the increased demands made upon the oxygen-carrying capacity of the blood.

Diurnal variations. Quite remarkable differences in percentage volume of erythrocytes were observed from day to day, and even from hour to hour. Numerous experiments in which horses were worked, fasted and then fed, allowed to go thirsty and then watered, all failed to account for the variations observed in jugular blood. The data obtained indicated that any change in the concentration of the blood, arising from these factors, is at most very transient. The real explanation of the variations, however, was at once found on tapping at different points in the circulation. In comparing blood from the jugular vein with that from the ear, it was found, with very few exceptions, that the latter gave a higher red count than the former, and was also subject to far greater variation. The difference between ear blood and jugular blood was greatest in animals feeding at rest, and least in animals excited or at work; an increase in one count being generally associated with a decrease in the other.

These observations indicated the mechanical state of the circulation as the important factor in determining distribution of erythrocytes throughout the body, and the few simple experiments recorded in Graph IV provided direct evidence of its operation.

Graph IV.



Showing the influence of the mechanical state of the circulation, upon the distribution of the red cells.

Fig. 1 of Graph IV represents the changes in red corpuscle count in an animal excited just prior to feeding; Fig. 2 those of an animal rested and fed immediately upon return from work; Fig. 3 those of an animal taken from the manger and threatened for 30 minutes by exhibition of a familiar whip. In all these cases the counts on peripheral and systemic blood converge most closely when the circulation is most active, and disagree most widely when the circulation is more sluggish. A less active circulation thus results in a concentration of the red corpuscles in the peripheral capillaries, possibly owing to relative increased lymph formation and possibly owing to sedimentation.

On many occasions it was also noticed that both ear and jugular red counts had increased or decreased together; observations which may be explained by assuming concentration or dilution in some other part of the circulation.

The general results, however, show that in horses the jugular blood is far more representative of the average circulation than is blood taken by ear puncture; that factors beyond the control of the observer may give very misleading results when ear blood is studied; but that even jugular blood is not free from variation.

Pregnant and nursing mares. The results obtained from a few animals of this class may be summarised by stating that no influence of gestation or lactation, as such, could be established. The red corpuscle counts varied greatly between individual mares, being always highest in those with a past history of active life, and such differences obscured possible variations centring around sex.

Foals. In foals the observed red count was very high just after birth, but in two months had decreased considerably. Two cases will serve to show that the most rapid fall occurs in stabled foals at rest:

	Percentage volume of red corpuscles	
Foal	At birth	After 2 months
(*a*) Permanently stabled	48	30
(*b*) Running with mother	42	36

Again the influence of physical activity is manifest.

Donkeys and Mules. The red cell picture of donkey and mule blood differs in an interesting way from that of the horse. The average diameter of the erythrocytes is approximately $6{\cdot}2\mu$ for both donkey and mule as against $5{\cdot}5\mu$ for the horse. For the donkey the ratio of percentage volume and numerical count was generally found greater than $5k$, while for mules it approximated $4{\cdot}35k$, the figure for the horse. The inference is that the corpuscles of the mule are thinner than those of donkey and horse.

The Leucocytes.

The classification of the leucocytes adopted in this work is that which is generally accepted to-day, as fully discussed by du Toit [1917] in his article on bovine blood. Equine leucocytes are not unlike those of bovines, as studied in stained smears, with the exception of the eosinophiles and basophiles. The granules of the latter cells are very large and form a characteristic feature of equine blood.

The *number* of leucocytes in the blood of healthy horses is very variable, not only as between individuals, but also in the same animal from time to time. The general range is from 5 to 20 thousand per cubic millimetre. In any individual animal the hourly and daily variations are not great, although exceptions are not uncommon.

Several experiments were undertaken to ascertain the influence of ordinary non-pathological factors upon the white count. Results may be summarised as follows:

(*a*) Moderate variations in supply of food and water showed no definite effects, and the jugular blood certainly reflected no evidence of any "digestive leucocytosis."

(*b*) Exercise always increased the white count of jugular blood, in some cases by over three thousand per cmm. On cessation of exercise the numbers often decrease again very rapidly, even to a figure below the pre-exercise count.

(*c*) Simultaneous observations upon ear and jugular blood yielded no conclusive results, except with exercise. In this case the results obtained indicate that the distribution of leucocytes also depends largely upon the mechanical state of the circulation.

To ascertain the extent to which technique of sampling influenced the white count, variations were made upon the usual procedure of first slapping the ear and collecting from a large puncture. A small puncture was made and blood obtained by squeezing at the base and at the apex. The following data represent one case:

	Leucocyte count
Ear, ordinary technique	$11{\cdot}3 \times 10^3$
Ear, squeezed at base	$7{\cdot}4 \times 10^3$
Ear, squeezed at apex	$14{\cdot}3 \times 10^3$
Jugular blood for comparison	$12{\cdot}4 \times 10^3$

From these marked variations it may be concluded that the white cells cling to the walls of the capillaries and the wound, especially if the blood flow is slow. When the circulation is rapid, or when the blood is forced out under considerable pressure, the leucocytes become detached, or are prevented from clinging, with consequent increase in apparent numbers. This explains why the ear white count is usually lower than the jugular.

Differential Counts. Differential leucocyte counts, obtained by the useful method (modified Ehrlich) already described, showed the following general figures:

Differential count of equine leucocytes	Lympho-cytes	Mono-cytes	Neutro-philes	Eosino-philes	Baso-philes
Horses, average of 7 each:					
(*a*) Stabled	36	4	54	5	1
(*b*) Medium	39	4	52	4	1
(*c*) Fast working	40	5	50	4	1
(*d*) General average	38	4	53	4	1
Normal variations	45–30	8–2	60–45	9–3	3–0
Extreme variations in 200 cases	50–25	9–0	62–54	15–1	3–0
Donkeys, average	53	4	34	8	1
Mules:					
(*a*) Clinically healthy	41	4	49	6	1
(*b*) Slight injuries	32	3	61	4	—
(*c*) Inoc. earlier against anthrax	52	3	39	6	1

The most interesting feature of this comparative table is the curious reversal of the figures for lymphocytes and neutrophiles, as between horses and donkeys. The horse shows lymphocytes 38 and neutrophiles 53 as general average, while the donkey shows neutrophiles 34 and lymphocytes 53. The count for mules approximates that of horses, when clinically healthy animals are considered. The slightly higher neutrophile count in group (*b*) mules may perhaps be accounted for by the slight injuries from which they were suffering. The high lymphocyte count in group (*c*) mules may perhaps be due to the fact that these animals had been inoculated against anthrax some months earlier.

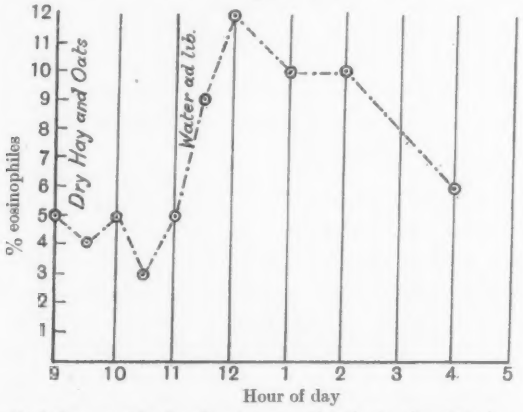
Breeding mares and foals.

To these data may be added a few observations upon mares and foals. Taken just before or after parturition, the mares showed a very high neutrophile count, even over 70 %. An even higher neutrophile count, about 80 %, was characteristic of foals shortly after birth. After about two months this neutrophilia had disappeared, provided the animals remained healthy.

Influence of other factors.

Various minor deviations from the usual mode of life did not appear to influence the differential count in any regular way; except for the eosinophiles, which were generally increased by water-drinking after a period of thirst or fasting. Graph V offers a typical example of transient eosinophilia following large consumption of water after dry feeding.

Graph V.



Showing the influence of food and water upon the % of eosinophiles in one horse.

General Discussion.

It is of interest to compare the findings recorded in this paper with the limited data already available in the same direction. References to healthy equine blood in the general haematological literature appear to be very scanty, and it is to be regretted that South African library facilities do not always allow of consultation of such references as can be tracked by abstract. Burnett quotes various authors as obtaining erythrocyte counts ranging from 6·3 to 8·5 million, and leucocyte counts varying between 5·6 and 15 thousand, for adult horses. The white count is approximately that recorded in this paper, but the red counts quoted by Burnett suggest that other workers have contented themselves too readily with animals of one class. The data now recorded show a much wider range, considerably below 6·3 million for permanently stabled horses and even up to 12 million for race-horses in hard training.

Frei [1909], working in this laboratory, compared the centrifuge and the counting chamber, obtaining 4·7*k* for the ratio between percentage volume and numerical count, and preferring the volumetric method for determining the proportion of erythrocytes. His ratio is thus about 8 % higher than the one now offered on a more extensive series of observations. The horses

available to Frei belonged to the same miscellaneous class as our own original lot, and showed variations from 22 to 43 for corpuscular volume. The astonishing thing is that no serious attempt was made to elucidate the nature of these differences, and the extraordinary effect of regular work seems to have wholly escaped his notice.

In regard to corpuscular dimensions, some authors give the average size of erythrocytes as 5·5μ, but others give figures as high as 5·8μ. The former figure is the average now established upon several hundred horses.

The differential counts quoted by Burnett for horses show higher neutrophile and lower lymphocyte percentages than those recorded in this paper. Stephens and co-workers [1921] have called attention to the variation in distribution of leucocytes in different parts of smears made in the ordinary way, and since our own technique has overcome this difficulty, the data now recorded would seem the more reliable.

By far the most striking observations recorded by us concern the remarkable influence of regular exercise upon the proportion of erythrocytes in horse blood. The available recent works upon horse blood all deal with pathological conditions, and nowhere does any reference seem to be made to the fundamental influence of normal work. In the various standard text-books upon Physiology and Haematology, the same omission is conspicuous. Dealing, as they do, largely with the human subject, in which normal variations are less noticeable, no stress is laid upon this factor, which plays so remarkable a rôle in the physiology of the horse. It is generally accepted that slight variations occur with mode of life, but whether or not these are specifically related to exercise is hardly discussed, although factors such as "altitude" are almost unduly stressed—perhaps because immediately detectable. Some workers on human blood have found that "robust" people have a higher, and "obese" people a lower, red count. The numbers are also lower for women, and it has been recorded that school children show a higher count at the end of a long vacation, than at the beginning. It is true that the variations generally recorded in healthy human individuals are registered in hundreds of thousands as against the tremendous variation of 4 million to 12 million between the stabled laboratory animal and the fully trained race-horse, but is it not possible that the minor differences in the human are due to differences in the amount of exercise rather than to other factors?

For the horse, the facts now recorded are perhaps not so surprising when considered in the light of its evolutionary history. The ancestor of the modern horse depended upon speed and endurance for its very existence; two qualities conditioned by a highly efficient circulatory system, and the demand for a high oxygen-carrying capacity of the blood. Is it then astonishing that the modern horse is born with a potential capacity for high speed and great endurance, and that during hard training his store of haemoglobin should be increased as fast as his muscles develop, so that finally more than half the total blood volume of a "finished race-horse" consists of erythrocytes?

In how far does this phenomenon of increase of red corpuscles with exercise, so strikingly shown here for the horse, apply to other animals? The problem at least seems a promising one for future investigation.

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LXXXII. THE FOOD VALUE OF MANGOLDS AND THE EFFECTS OF DEFICIENCY OF VITAMIN A ON GUINEA-PIGS.

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From the Wellcome Physiological Research Laboratories.

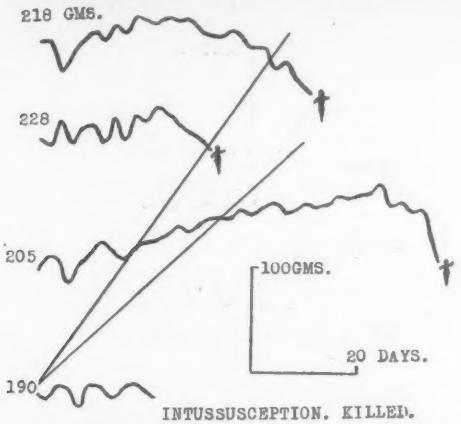
(*Received October 11th, 1922.*)

Our colleagues Glenny and Allen [1921] presented to the Pathological Section of the Royal Society of Medicine an account of an investigation into an epizootic amongst a stock of guinea-pigs. They presented conclusive evidence that the epizootic could be entirely controlled by alteration of the diet. The diet which resulted in the outbreak consisted of bran, oats, water and mangolds. Substitution of the mangolds by cabbage, grass or lucerne stopped the epizootic. When guinea-pigs in adjacent runs were fed on the two diets, those on mangolds were attacked, whilst those with grass remained healthy, although no precautions whatever were taken to prevent infection of the healthy animals. Isolation was not carried out, the runs were not disinfected, and the attendants handled both groups of animals indiscriminately. These results are of such importance that the following attempt was made to determine the factors or deficiencies in the mangold which rendered the guinea-pigs sensitive to the epizootic. A few experiments were done by Glenny in which an alcoholic extract of carrot was given to the guinea-pigs with a view to supplying a possible deficiency of vitamin A. These indicated that the supply of vitamin A, although it reduced the incidence of the disease, did not stop it, and the guinea-pigs did not put on weight.

The following experiments were begun after the epizootic had been controlled by dietary means, and the effect of the diet was re-investigated.

Chart I shows the weight curves of four guinea-pigs fed on unlimited bran, oats, mangolds and water, the animals eating about 40 g. of mangold each per diem, and Chart II those of guinea-pigs kept on a control diet of bran, oats, autoclaved milk and greenstuff. These latter gained weight rather faster than Miss Hume's [1921] "standard" guinea-pigs (43·7 % in 25 days, as against 34·2 % in Miss Hume's experiments).

It was found that all the guinea-pigs placed on the mangold diet eventually succumbed within a period of about two months. In Chart I, the guinea-pigs were about 200 g. weight when started. In Chart III, they were younger—about 150 g.—and the younger guinea-pigs succumbed earlier than the older ones.



The charts are drawn to different scales indicated in each case. The oblique straight lines on each chart represent the greatest and least rate of growth shown in the experiment with a normal diet represented in Chart II. The abscissa for each curve is at a different level; the figure at the beginning of each curve shows the initial weight of the guinea-pig in grams.

Chart I. Effect of basal diet of bran, oats, and mangolds. One of the animals had an intussusception and was killed. We have met this condition fairly frequently in animals under 200 g. in weight which have been deprived of greenstuff.

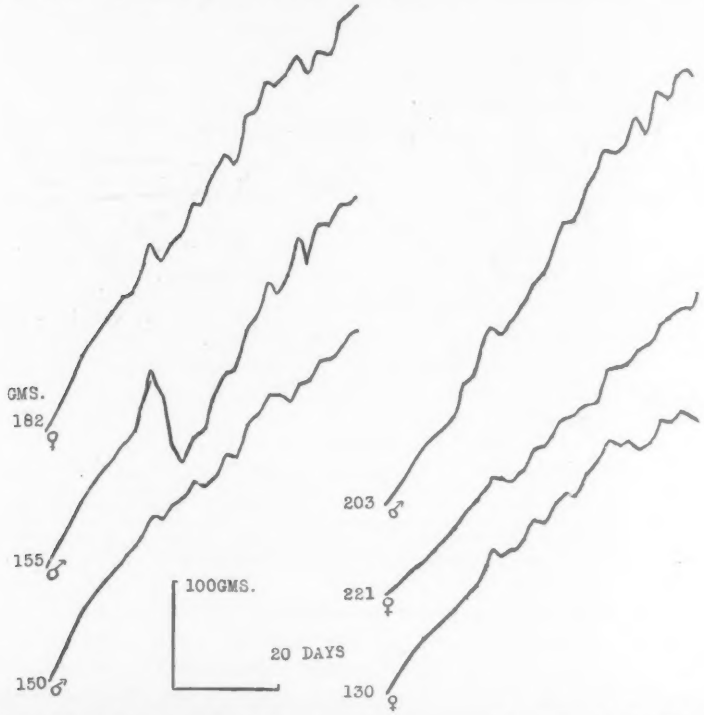


Chart II. "Normal" diet consisting of bran, oats, lucerne, grass and autoclaved milk. This diet was used as a control for some experiments on scurvy. We have obtained since writing this paper even better results than these, with the "synthetic" diet described later.

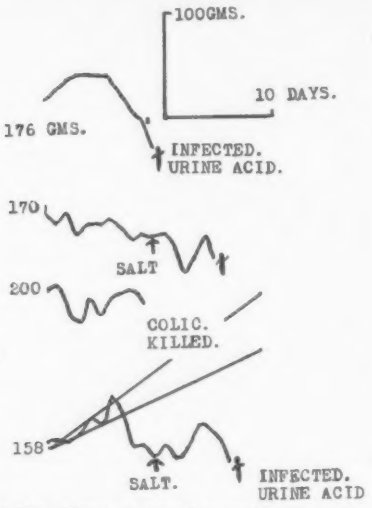


Chart III. Effect of basal diet of bran, oats, and mangolds on younger guinea-pigs. It will be seen that these animals died sooner than those in experiment 1.

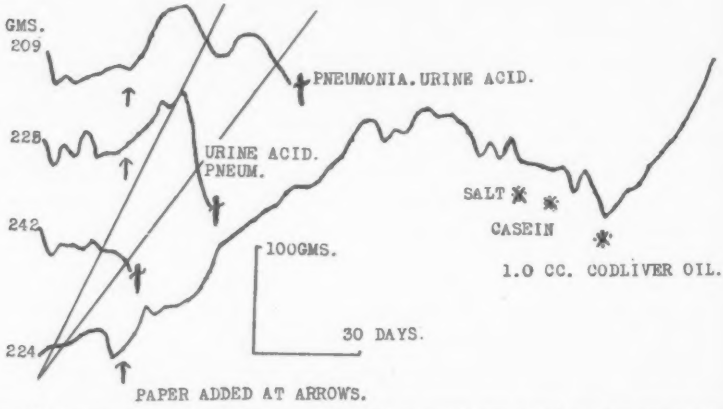


Chart IV. Guinea-pigs on basal diet as in I and III, to which was added packing paper at the arrows. One of the animals died the same day, the others started to grow again, one growing for over two months before decline set in. As the result of experiments which had been going on in the meanwhile, salt mixture (calcium lactate and sodium chloride), caseinogen and cod-liver oil were added to the diet (see further charts). It will be seen that the limiting factor in this guinea-pig was the absence of something supplied by cod-liver oil—presumably vitamin A.

It was noticed whilst weighing the animals on the deficient diet, that they eagerly nibbled at any pieces of paper or cardboard which they could reach; so clean white packing paper was placed in the cages. The guinea-pigs each consumed about 5 g. of paper every day. Chart IV indicates the effect on

the growth, and it will be seen that out of four guinea-pigs, one died on the first day that the paper was given, but the other three gained in weight as a result, and one of the three continued to gain for over two months before a decline set in. Suggestions are put forward below as to the explanation of this.

Post-mortem examinations revealed several abnormalities in the guinea-pigs whilst on the mangold diet.

As in Glenny's experiments, there was constantly some sign of an infection present, either

(*a*) Alkaline stomach contents.
(*b*) Pneumonic consolidation or abscess of the lung.
(*c*) Purulent pericarditis.
(*d*) Enteritis.

When the urine was tested during life and post-mortem, it was always found to be strongly acid, and therefore quite free from the normal precipitate of phosphates which renders the urine of the healthy guinea-pig turbid. This observation led to the suggestion that the mangold, bran and oats diet is deficient in certain basic constituents, and following the indication of McCollum's feeding experiments on rats [1920] (from which he concludes that roots and seeds, besides other deficiencies, lack the three inorganic elements calcium, sodium and chlorine), it was decided to add a salt mixture to the bran, oats and mangold diet. A mixture of calcium lactate and sodium chloride was made and mixed as thoroughly as possible with the bran and oats, about 1·5 g. of calcium lactate and 1·0 g. of sodium chloride per guinea-pig per diem being given. This resulted in a return of the urine to the normal alkalinity, when examined during life and post-mortem, and improved the growth curves. (First part of Chart V.)

In connection with this, we made an examination of the ash, by drying mangold at 100°, and then incinerating in a muffle furnace. Magnesium, sodium, potassium and iron were found to be present, but no qualitative test for calcium could be obtained, and in attempting to estimate calcium by Cahen and Hurtley's method [1916], no potassium permanganate was used up in the final titration.

The beneficial effect of the salt mixture is therefore probably due to its supplying the extra calcium. The packing paper also helps in the same direction, for analyses of the ashed paper showed that there is a certain amount of calcium present:

Percentage of ash in paper ...	...	1·7 %	1st determination.
,, ,, ,, ,, ...	...	1·6 %	2nd ,,
,, ,, calcium in paper	...	0·126 %	1st ,,
,, ,, ,, ,,	...	0·190 %	2nd ,,

Using Sherman and Gettler's tables [1912], we have calculated that the calcium supplied by the 5 g. of paper eaten per guinea-pig per diem would

be about equal to that supplied by 6 cc. of cow's milk per diem, and is about equal to half the calcium in 40 g. of cabbage (Miss Hume's standard amount).

Besides supplying this small amount of calcium, it is possible that the paper may act as "roughage" in the diet, the alimentary tract of the guinea-pig being only suited to an extremely bulky diet, and requiring a comparatively large amount of ballast in order to secure normal intestinal movements. It is, however, very difficult to believe that a guinea-pig devouring 40 g. of mangold per diem is in need of any further cellulose, and we are inclined to the opinion that the paper acts chiefly by partially supplying the calcium deficiency. Mangold, though deficient in calcium, contains about the same percentage of potassium as cabbage.

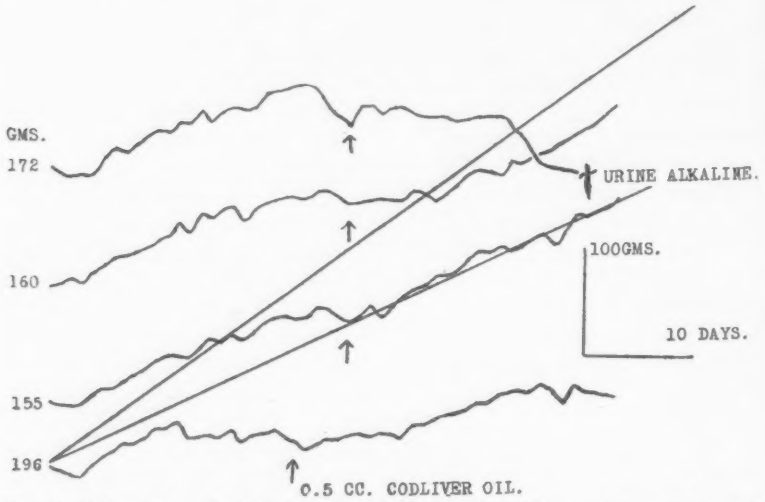


Chart V. Guinea-pigs on basal diet as in I and III, plus salt mixture and paper. 0·5 cc. cod-liver oil per guinea-pig per diem administered at the point marked by the arrows. One guinea-pig only then approached a normal rate of growth, the deficiency of protein probably being the limiting factor in the case of the other three guinea-pigs, one of which ultimately succumbed.

An important point in connection with the first part of Chart V arises in consideration of the changes in the acidity of the urine.

On the mangold diet, the urine is acid. On a greenstuff diet, the urine is alkaline. 40 g. of cabbage, according to Miss Hume's results, is sufficient for normal growth in guinea-pigs. If, however, a comparison is made between the ash of mangold and cabbage, it will be found that whereas 40 g. of ashed mangold give sufficient base to neutralise 35·2 cc. $N/10$ acid (this is obtained from a direct titration of the ash—is probably therefore too high because of the loss of sulphur, but the error is only of the order of 2 or 3 cc.), 40 g. of ashed cabbage will neutralise 42·6 cc. $N/10$ acid (Sherman and Gettler, calcu-

lated), not a significant difference. There are two explanations that offer themselves:

(1) The potassium salts of the mangold are not absorbed by the intestinal mucous membrane in the absence of calcium salts to balance their toxic effects. It is a commonplace that potassium poisoning is very difficult or impossible to produce by oral administration of potassium salts. As a result, the acid-base balance is disturbed—the animal's only source of salts is the bran and oats, the ash of which is acid.

(2) The deficiency of calcium may lead to a disturbance of the metabolism, possibly secondarily to an infective process, which leads to excessive breakdown of body protein, and the consequent production of abnormal amounts of sulphuric and phosphoric acids in excess of the neutralising value of the food salts absorbed.

The experiments so far done are in favour of the first explanation, for animals that died as a result of other deficiencies in diet, but with a good supply of calcium lactate, mostly had an alkaline urine, whereas animals fed on the diet without mangold, and with no other source of vitamin C, although supplied with calcium lactate, developed an acid urine.

This is only one of many indications of the need for further investigation of the physiological nature of the disturbances of the animal economy by a deficient diet, and is one which we are further examining.

In Chart V, four guinea-pigs were started on an initial diet of mangolds, bran and oats, salt mixture and paper, and it will be seen that the salt mixture and paper did not sufficiently supplement the diet to give continued normal growth curves, and after about a month, all the guinea-pigs started to decline in weight. The similarity of the post-mortem lesions to those found in rats fed on diets deficient in vitamin A suggested the addition of some source of vitamin A. This had been attempted before with carrot extract prepared by Zilva's method, but without much success, for other deficiencies of the mangold were not made up. We now tried cod-liver oil instead of the carrot extract and 0·5 cc. per guinea-pig per diem was administered by hand. This was found to cause a great improvement, and the guinea-pigs, with only one exception, resumed a fair rate of growth.

It follows, therefore, that the mangold, bran and oats diet, besides being deficient in calcium and perhaps roughage, is also lacking in the fat-soluble vitamin A. Our impression is that the dose of 0·5 cc. of the oil used in this experiment for our guinea-pigs was marginal, which may account for the failure of one of the four guinea-pigs to survive on the oil-supplemented diet. We have frequently noted that rats kept for a prolonged period on a diet deficient in vitamin A seem to undergo some permanent change, which renders them unresponsive to any treatment with cod-liver oil. It is possible that guinea-pigs also develop a similar condition of unresponsiveness, when deprived of the vitamin for prolonged periods of time.

But further feeding experiments with another set of guinea-pigs on a diet

of bran and oats, mangolds, salt mixture, paper and oil, revealed a further deficiency in the diet, viz. protein.

Chart VI shows the weights of two guinea-pigs which were started with a diet of bran, oats, mangolds, paper and salt mixture, and then when a decline in weight, due to deficiency of fat-soluble A, occurred, oil was given, with a result that growth was again resumed. After about a month of this supplemented diet, however, a fresh drop in the weights of both guinea-pigs occurred, and one of them succumbed, but the other guinea-pig quickly picked up weight again on adding caseinogen to the diet. This guinea-pig continued a normal rate of growth, and was still continuing to grow at the time of writing, so that it is concluded that a fully supplemented diet has been attained. (See also the latter part of Chart IV.) The question arises whether the caseinogen acts beneficially by:

(*a*) Supplementing the phosphorus intake.

(*b*) Supplementing deficient protein, either a deficiency in certain amino-groupings, or a deficiency in total amount.

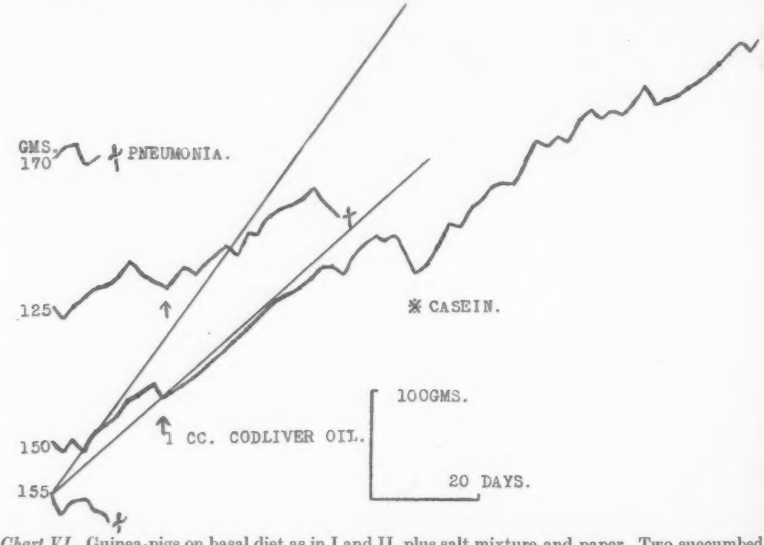


Chart VI. Guinea-pigs on basal diet as in I and II, plus salt mixture and paper. Two succumbed before administration of oil was commenced (at arrows). Of the two survivors, one eventually succumbed to protein deficiency, but the other quickly resumed a normal rate of growth on the addition of caseinogen to the diet (at star).

Osborne and Mendel [1918] have shown with rats, that a shortage of phosphorus leads to a considerable slowing in growth followed by a fall in weight, but on adding caseinogen to the phosphorus-free diet, an improvement was made, and still greater improvement when inorganic phosphorus was added. The addition of edestin (a phosphorus-free protein), however, only led to complete cessation of growth and a decline.

The 12·5 g. of bran and oats eaten in a day contains 0·05 g. of phosphorus [Sherman and Gettler, 1912], whereas 40 g. of cabbage only adds 0·013 g. of phosphorus, an amount which could be easily covered by a small extra consumption of bran and oats, such as often takes place. We are led to conclude that, since cabbage can fully supplement a bran and oats diet, the addition of phosphorus is probably not the influencing factor, but that both caseinogen and cabbage supply a certain amino-acid or acids not present in bran and oats or in mangolds. This is a rather remarkable conclusion, considering the small amount of nitrogen in greenstuff. It is paralleled by the observations of Thomas that the N of potato is especially effectual in supplying the N requirements of the body.

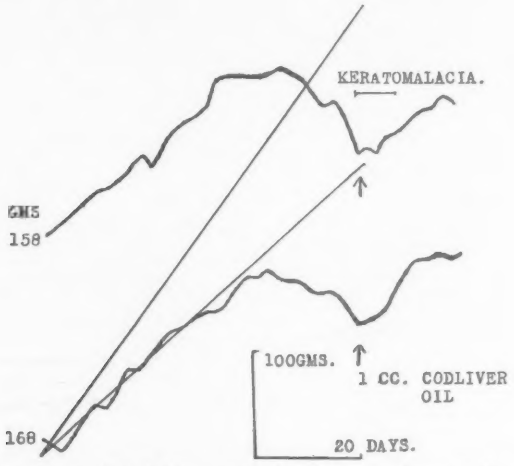


Chart VII. Guinea-pigs on diet of bran, oats, salt mixture, caseinogen and mangolds. After definite symptoms of vitamin A deficiency had become apparent, cod-liver oil was administered, at the arrows. Duration of the keratomalacia marked by straight line.

Two guinea-pigs, Chart VII, were started on a diet of bran and oats, caseinogen, salt mixture, paper and mangolds, and it will be seen that this diet was complete with one exception, viz. fat-soluble A. After a good initial growth for over a month, both guinea-pigs dropped in weight considerably, and one of them developed keratomalacia. There was a clouding of the cornea of one eye, which gradually became opaque. No haemorrhagic discharge was seen, and no conjunctivitis. In the Report of the Medical Research Committee [1919], it is stated that in rats it is the swelling of the eyelids and conjunctivitis which appears first, and this, if untreated, leads to thickening and clouding of the cornea, and ultimate blindness. With guinea-pigs, however, we have found that the corneal clouding always appears first, and several times we have received guinea-pigs from stock with completely opaque corneas, which have gone on to panophthalmia. We have never observed any haemor-

rhagic or purulent discharge preceding the keratomalacia in guinea-pigs. In rats, we have sometimes observed the uncomplicated corneal change first, but more frequently conjunctivitis precedes the corneal cloudiness.

When the guinea-pigs had each lost about 50–100 g., and the keratomalacia had developed unmistakably in the one, 1 cc. of cod-liver oil was given to each guinea-pig every day, which resulted in an almost immediate increase in weight, and a resumption of a normal rate of growth. There was a complete disappearance of the eye trouble after about one week's feeding with the oil.

We have confirmed the absence of fat-soluble A from mangold by feeding experiments with rats (Chart VIII).

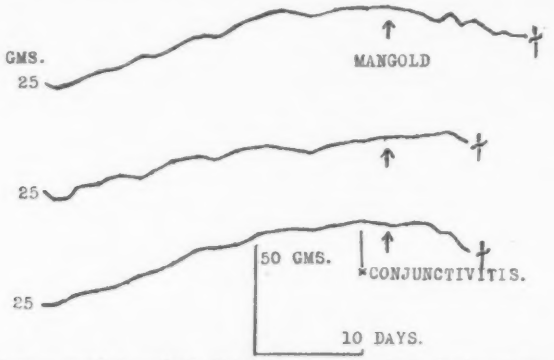


Chart VIII. Rats on purified synthetic diet deficient in vitamin A. Conjunctivitis developed at point marked by cross, and here mangold was added to the diet. No improvement in the condition and weight of the rats resulted, and they all eventually died.

The fact that our guinea-pigs developed no symptoms of scurvy whilst on the mangold diet supplemented as above, for a period extending over more than two months, indicates that the mangold supplies an adequate amount of the anti-scorbutic vitamin C. Vitamin B is supplied in sufficient quantity by the bran and oats mixture, and is also present in the mangold.

Our experiments therefore lead to the conclusion that mangold is deficient in the following: (1) calcium, (2) fat-soluble A, (3) protein, (4) ? roughage; but contains a sufficiency of the anti-scorbutic vitamin to keep guinea-pigs free from scurvy. This confirms the rule laid down by McCollum for seeds, roots and tubers generally.

The only essential dietary substance added by the mangold to bran and oats is vitamin C, although there is of course some energy value in the various other constituents, and by feeding experiments on rats we have shown that there is a fair quantity of vitamin B present.

Once again, as other observers have noted, vitamin A deficiency accompanies a low calcium content, so that it can almost be laid down as a general rule that where there is vitamin A deficiency, calcium deficiency probably

runs parallel with it; and conversely, where there is a good supply of the fat-soluble vitamin, there is often a high percentage of calcium, as the following figures taken from Sherman and Gettler's tables show:

		Vitamin A	Calcium content %
I.	Egg yolk	+	0·143
	Cow's milk	+	0·124
	Beans (dried)	+	0·165
	Nuts	+	0·270 (almonds)
II.	Egg white	−	0·011
	Fish (white)	−	0·022
	Potatoes	−	0·006
	Rice	−	0·006–0·01

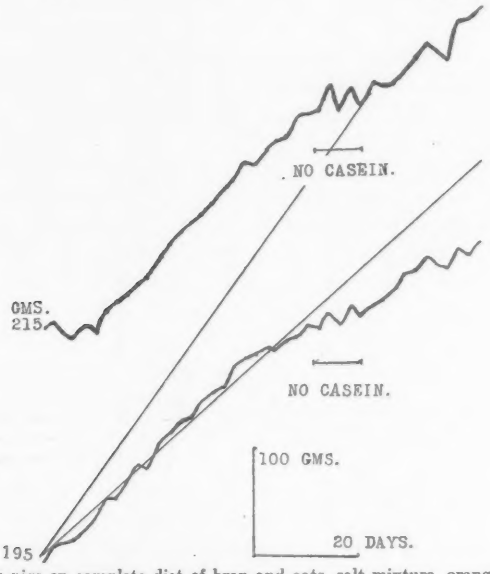


Chart IX. Guinea-pigs on complete diet of bran and oats, salt mixture, orange juice, cod-liver oil and caseinogen. Flattening of the growth curves when caseinogen was withdrawn from the diet for a period marked by the horizontal line.

We have made various efforts to feed guinea-pigs on a basal artificial diet similar to that used in feeding experiments on rats, but made up according to calculations based on analyses of bran and oats, milk and grass, with the idea of supplementing this basal diet with the three vitamins A, B and C in turn. All our experiments, however, have been brought to an untimely conclusion by the fact that the guinea-pigs refuse to eat any of the artificial diet.

Starting with bran and oats as a basal diet, and supplementing this with heated caseinogen, salt mixture as above, and paper, one can, however, study the effects of the vitamins A and C in the guinea-pig in a more or less uncomplicated way.

Two guinea-pigs were placed on this "synthetic" diet, as shown in Chart IX, bran and oats, heated caseinogen, salt mixture and paper being given, together

with 3 cc. lemon juice and 0·3 cc. cod-liver oil per guinea-pig per diem. After a time, however, 5 cc. orange juice was substituted for the lemon, as it was found that the guinea-pigs were averse to the sour taste of the lemon, whereas they drank the orange juice eagerly. It will be seen from the curves that a very satisfactory rate of growth can be maintained on this diet. A flattening of the growth curves occurred when caseinogen was withdrawn from the diet for a short period, thus emphasising the need of supplementing the protein of the bran and oats. It is, of course, of great importance that the temperature of the animal house in which the guinea-pigs are kept should be kept as constant as possible.

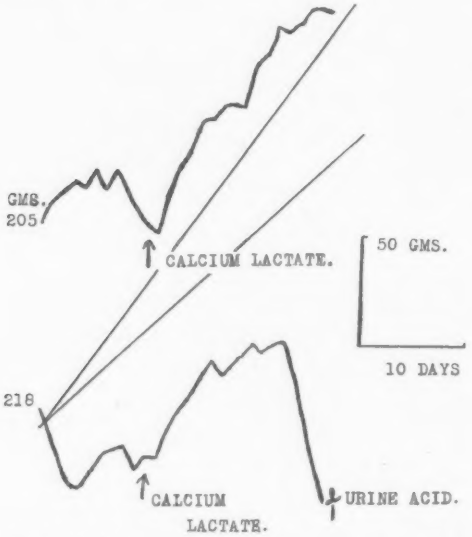


Chart X. Showing effect of calcium deficiency. Guinea-pigs on bran and oats, mangold and cod-liver oil. (Paper and caseinogen were omitted because of the possibility of their containing small quantities of calcium.) A rapid decline set in, from which both animals quickly recovered on the addition of calcium lactate to the diet. One guinea-pig later succumbed to protein deficiency.

We are unable to confirm Miss Hume's statement [1921] that guinea-pigs have an intolerance for unemulsified fat, for we have performed a large number of experiments in which we have fed guinea-pigs with cod-liver oil, and in every case the oil has had a beneficial effect provided other essential food factors are not missing. We would suggest that Miss Hume's failure to obtain growth on oil was probably due to the basal diet of bran and oats and orange juice being inadequate, so that when the test substance, *e.g.* butter-fat or oil, was added to the diet, the beneficial effect of the addition of vitamin A was masked by the protein and inorganic deficiencies. For example, in Chart X, guinea-pigs were put on a diet of mangolds, bran and oats and cod-liver oil, and very soon a decline in weight occurred, which might have been ascribed

to an intolerance for oil on the part of the guinea-pig. That this was not the case was shown by supplying the deficient calcium of the dietary, when a quick recovery of the weight of the guinea-pigs was obtained, and they continued growing satisfactorily for some time, the oil still being continued, until finally, the deficiency of protein became apparent.

It is obvious that the need of the guinea-pig for an adequate supply of calcium is an urgent one, and the effects of its absence show themselves earlier than those of the vitamins. Vitamin A deficiency takes about 40–50 days to become apparent, and scurvy takes about three weeks to develop. On a diet containing inadequate calcium, however, guinea-pigs lose weight in a few days (see Chart X).

These experiments fully confirm Miss Hume's conclusion that guinea-pigs require a large amount of vitamin A, and they have the advantage that the addition of vitamin A to the diet was made in the form of cod-liver oil—much less admixed with other essential substances than in the case of the greenstuff and milk studied by her. The effect of deprivation of vitamin A is much more regularly obtained in guinea-pigs than in our own stock of rats, several of which grow for long periods on a deficient diet and even continue to grow whilst developing obvious eye changes. The larger dose of oil necessary for the guinea-pigs also suggests that they may be more suitable than rats for the estimation of vitamin A, and we are making some experiments with that in view.

Conclusions.

(1) The deficiencies of a diet of mangold, bran and oats and water which was the controlling factor in an epidemic amongst guinea-pigs have been investigated.

(2) Vitamin A and calcium salts have been shown to be deficient, and the protein to be deficient in quantity or composition or both.

(3) Keratomalacia in the guinea-pig as a result of vitamin A deficiency has been observed and cured by the administration of cod-liver oil.

(4) The administration of cod-liver oil to guinea-pigs has been shown to be an adequate means of administering vitamin A, and to be well tolerated by guinea-pigs if the diet is otherwise satisfactory.

(5) Direct confirmation of the necessity for vitamin A in the diet of guinea-pigs, inferred by Miss Hume, has been obtained.

(6) Another instance is provided of a foodstuff in which a deficiency of vitamin A is accompanied by a deficiency of calcium.

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LXXXIII. THE CATALYTIC DESTRUCTION OF CARNOSINE IN VITRO.

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(*Received October 20th, 1922.*)

In a previous paper [Clifford, 1922] an account was given of the destruction of carnosine in beef and rat muscle kept in cold storage. Since the temperature at which the destruction occurred was at or below zero, it was improbable that it was due either to enzyme or bacterial action. The time taken however was prolonged—6–10 months—and therefore a slow change due to an enzyme or to bacteria might have been the cause. In order to exclude these possibilities experiments were carried out at 100°. A similar though far more rapid disappearance of carnosine took place at this temperature pointing to a simpler catalyst than an enzyme, while bacterial life is also inconsistent with a temperature range of 0°–100°.

Experimental Results.

(1) *Destruction of carnosine in beef at* 100°.

From 3–5 g. of minced lean beef were weighed into test-tubes and 10 cc. water added. The tubes were then plugged with cotton wool and placed in a water-bath kept at 100°. When necessary more water was put in the tubes to prevent drying of the meat.

At intervals of 1–4 days a tube was removed, the contents made up to 95 cc. with distilled water and 5 cc. of 20 % metaphosphoric acid added to precipitate proteins. The carnosine present in the filtrate was estimated colorimetrically by the method described in a previous paper [Clifford, 1921, 1].

The numerical results of four experiments were as follows:

Days at 100°	*S*	15	29	29 *A*
Start	0·96 %	0·99 %	1·05 %	1·05 %
1	—	0·80	—	—
2	0·70	—	0·90	0·89
4	—	—	0·80	0·74
5	0·60	0·66	—	—
7	0·54	0·60	0·68	0·65
10	—	—	0·60	0·64
12	0·53	—	—	—
14	—	0·60	0·55	0·57
16	0·30	—	—	—
17	—	0·40	0·43	0·40
21	—	0·33	—	—

These results, Fig. 1, indicate the presence of a catalyst which removes carnosine from muscle. This removal is considerably hastened[1] by a rise of temperature and is therefore probably a chemical and not a physical change. On comparing the results with those previously published [Clifford, 1922] it is found that for the percentage of carnosine to fall to the same level 9–10 months is necessary at 0° as against 21 days with a temperature of 100°.

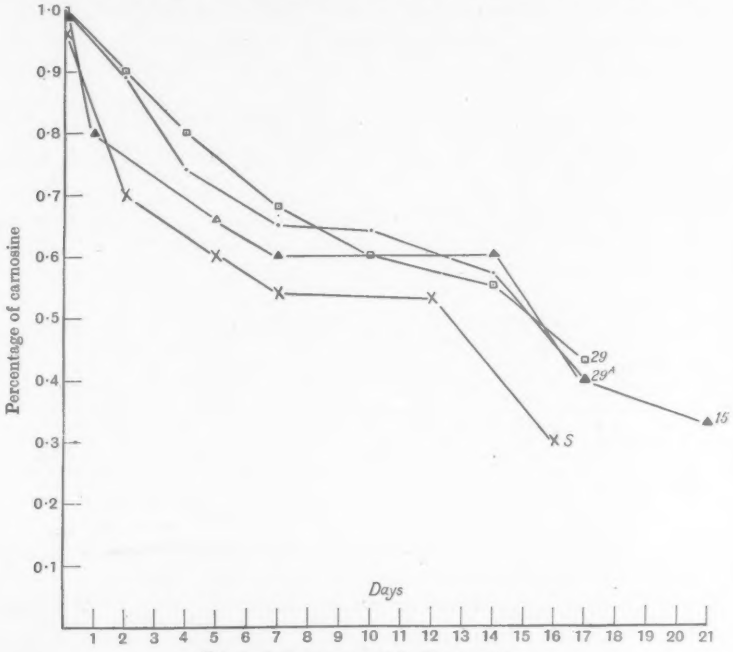

Fig. 1. 3–5 grams meat in 10 cc. water.

(2) *Catalyst not removed by boiling.*

Experiments were performed to find whether the catalyst were present in an extract made by boiling beef with water. Minced lean beef was put into a flask with water and boiled in a water-bath for 30–60 minutes. It was then filtered, and the residue again boiled with water for a similar period. The filtration was repeated and the two filtrates united. An estimation of the carnosine percentage of the extract was made, and portions of 10 cc. put in test-tubes plugged with cotton wool. The tubes were then placed in a water-bath at 100°.

In five experiments lasting 13–26 days no change took place in the carnosine content of the extract. Similar results were obtained in two other 13 day experiments, one kept at 60°, and the other at 37° after sterilisation by boiling.

[1] The coefficient is 1·5 for 10° rise whilst for physical actions it is generally 1·03–1·05.

Since the carnosine content of the extract in these experiments remained unchanged, the catalyst cannot be removed from beef by extraction with water for 1–2 hours at 100°.

(3) *Catalyst remains in beef after boiling.*

A series of test-tubes was taken containing 10 cc. of beef extract prepared as above together with 3 g. of boiled minced beef which had been washed in cold running water for 15 minutes to remove traces of extractives. The result as expected was identical with that obtained with fresh beef in water.

(4) *Catalyst removed by prolonged washing of boiled meat.*

Further experiments were carried out to find the result of prolonged washing of the boiled meat.

Five experiments with series of tubes containing 10 cc. of extract and 3 g. of boiled meat washed 3, 18, 24, 48, 48 hours respectively were kept at 100° with the following results:

	21 3 hours' washing	20 18 hours' washing	19 24 hours' washing	30 48 hours' washing	9 48 hours' washing
Days	%	%	%	%	%
Start	1·0	1·0	1·05	0·99	0·98
3	0·9	1·0	1·0	0·99	0·98
6	0·9	0·9	1·0	0·99	0·98
8	0·45	0·6	0·77	0·99	0·98
10	0·45	0·6	0·70	0·99	0·98
13	0·40	0·55	0·65	0·99	0·98
23	0·35	0·35	0·50	0·99	0·98
27	0·30	0·33	0·48	0·99	0·98

From these results it appears that the catalyst present in boiled beef which destroys carnosine can be removed by prolonged washing in running water, probably by separation of an adsorption compound from the muscle protein.

In one experiment the preliminary boiling of the meat was omitted and fresh unboiled minced beef was washed in running water for 24 hours. Three g. of this washed meat was then added to each of a series of tubes containing 10 cc. extract at 100°. The result was a disappearance of carnosine similar to that given by boiled beef washed for three hours.

Start	1·0 %	10 days	0·40 %
3 days	0·95 %	13 ,,	0·40 %
6 ,,	0·80 %	23 ,,	0·25 %
8 ,,	0·45 %	27 ,,	0·25 %

These results, Fig. 2, indicate that the catalytic destroyer of carnosine is present in some physical combination with the protein of beef muscle and may be removed by prolonged washing in cold running water. This removal is facilitated by boiling the meat before washing, though mere boiling for 1–2 hours does not extract the catalyst from the muscle.

The curve of disappearance of the base is peculiar and it is difficult to account for the delay in the start of the reaction with washed meat and extract as compared with fresh meat and water. In all the curves there is a sharp fall between the 6th and 8th days followed by a stationary period and then a slower steady fall.

This three-step curve is indicated in experiments on fresh meat and water (Fig. 1) and is strongly marked in experiments with cod muscle and with liver. A paper previously published [Clifford, 1922] shows a similar curve from beef kept in cold storage.

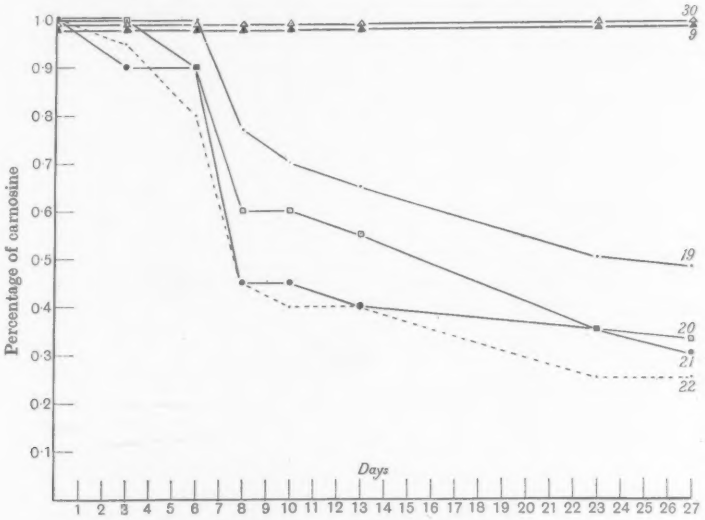

Fig. 2. 10 cc. meat extract + 3 grams washed meat.
30, 9. Meat boiled 1–2 hours then washed 48 hours.
19. " " " 24 "
20. " " " 18 "
21. " " " 3 "
22. Meat unboiled but washed 24 hours.

(5) *Presence of catalyst in muscles of white fish.*

Carnosine has been shown [Clifford, 1921, 2] to be absent from the muscles of white fishes. A series of experiments was performed using 3 g. of fresh minced cod muscle in beef extract in place of 3 g. of boiled and washed beef. Unexpectedly it was found that the carnosine disappeared giving a similar curve to that with washed beef in its peculiar three-stepped nature, though the actual position of the stationary period varies (Fig. 3).

Three experiments were carried out at 60° after preliminary sterilisation by boiling and three were kept constantly at 100°.

Days	*P* 60° %	*H* 60° %	7 60° %	*E* 100° %	14 100° %	24 100° %
Start	0·96	0·99	0·99	0·99	1·04	1·0
1	—	—	—	—	0·80	—
2	0·90	0·90	—	0·90	—	—
3	—	—	—	—	—	1·1
4	—	—	0·95	—	—	—
5	0·85	—	—	—	0·80	—
6	—	—	—	—	—	0·75
7	0·58	0·80	0·72	0·90	0·55	—
8	—	—	—	—	—	0·60
9	—	—	—	—	—	—
10	—	0·80	—	0·88	—	0·46
11	—	—	0·60	—	—	—
13	0·58	—	—	—	—	0·46
14	—	0·66	0·55	0·50	0·50	—
16	—	0·66	—	0·44	—	—
17	0·52	—	—	—	—	—
18	—	0·44	0·55	—	0·45	—
20	—	—	—	—	—	—
21	—	0·40	0·50	0·40	0·35	—
23	—	0·35	—	—	—	—
25	—	0·30	—	—	—	0·30
28	—	—	0·40	—	—	—

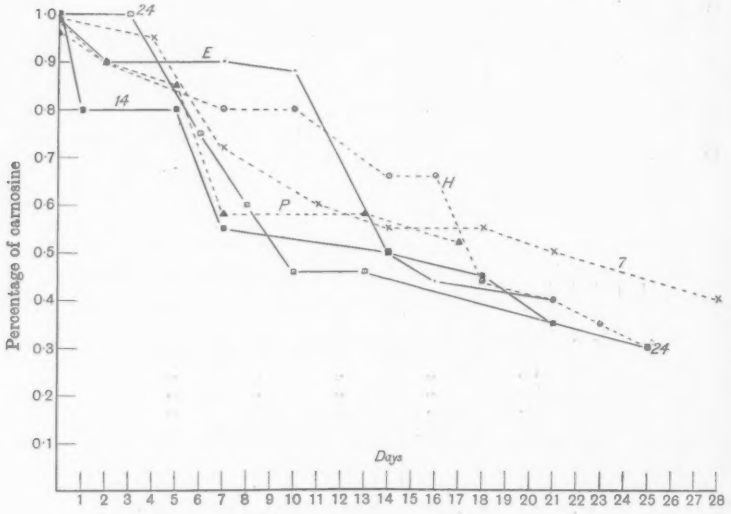

Fig. 3. 10 cc. meat extract + 5 grams cod muscle.
Unbroken lines 100° C. constantly.
Broken lines 60° C. constantly after sterilisation by boiling.

(6) *Presence of catalyst in liver.*

An experiment was performed using tubes with 10 cc. of beef extract and 3 g. of calf liver kept constantly at 100°. Carnosine estimations again gave a stepped curve of disappearance of the base.

A second experiment in which 1 g. of freshly killed rat's liver was used in place of the 3 g. of calf liver, and in which the temperature was 60° in place

of 100°, also showed a loss. As was expected from the lower temperature and smaller amount of tissue taken, the rate of disappearance was slower than in the first experiment (Fig. 4).

Days of experiment	16 100° and 3 g. liver. %	*Q* 60° and 1 g. liver. %
Start	1·0	0·96
1	0·80	—
2	—	0·90
5	0·55	0·77
7	0·55	0·77
13	—	0·64
14	0·50	—
17	0·42	0·60
21	0·25	—

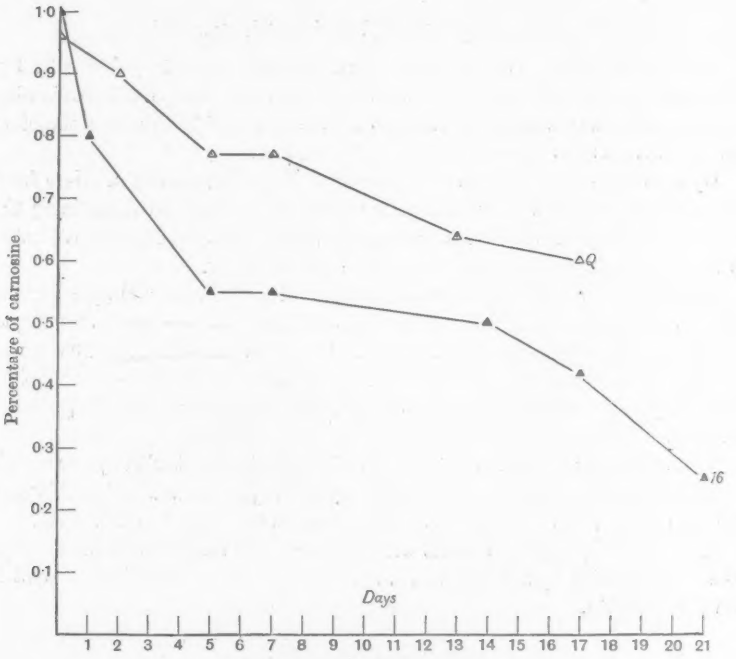

Fig. 4. 10 cc. meat extract + liver.
Q 1 g. rat liver kept at 60° C.
16 3 g. calf liver kept at 100° C.

A third experiment lasting 18 days, in which 3 g. of frozen calf liver was used as a source of the catalyst, showed no loss of the base. These findings point to the presence of the carnosine-destroying catalyst in liver as well as in muscle, whilst prolonged keeping in cold storage destroys the catalyst itself.

(7) *Absence of catalyst from kidney.*

Since skeletal muscle and liver had both been shown to cause the disappearance of carnosine from muscle extract, it was possible that the catalyst was present in all cells. Therefore an experiment was carried out using 1 g. of freshly killed rat's kidney, in place of liver or muscle, in 10 cc. of beef extract. The tubes were kept at 60°. No change in carnosine content took place till the 21st day, when a very slight fall took place. This fall however lay well within the limits of experimental error.

Similar results were obtained using 3 g. of sheep's kidney, 3 g. of bullock's kidney and 3 g. of frozen bullock's kidney, all kept at 100° for at least 20 days.

There is, therefore, no carnosine-destroying catalyst in the kidney, and consequently the possibility of existence of this catalyst in all cells cannot be maintained.

(8) *Absence of catalyst from invertebrate muscle.*

A series of test-tubes containing 10 cc. of beef extract together with 3 g. of fresh lobster muscle was left in a bath at 100°. No change in the carnosine content of the tubes was observed up to the end of 46 days, and therefore the catalyst was not present in the lobster muscle.

Oysters were next used as a further type of the invertebrata. Here from the first day onwards no pink colour could be obtained on diazotising the protein-free filtrate. However a deep yellow colour resulted and if estimated as carnosine showed no loss of base up to the 23rd day.

In all other experiments, *e.g.* with liver and muscle, or in simple keeping of meat in cold storage, disappearance of the base coincided with a less depth of colour, and not an alteration of hue. Therefore the lack of red colour on diazotising the oyster-beef extract was probably due to the presence of an inhibitory substance similar to that shown to exist in salmon muscle [Clifford, 1921, 2].

Unfortunately there was not enough of the beef extract with oyster muscle to use the precipitation method of Dietrich [1914] to show whether this were the case or not, but experiments are in progress to determine this point.

The definitely negative result with lobster muscle and the probably negative findings with oyster point to a lack of carnosine-destroying catalyst in the invertebrata.

Discussion of Results.

The experiments described in this paper indicate the presence of a substance which is capable of destroying carnosine in muscle extracts, and owing to its temperature range it must be a relatively simple catalyst.

It is not known whether the absence or lessening of red colour on diazotising filtrates from meat kept several days in water at 100° is due to actual destruction of the iminazole ring, or to a synthesis which prevents coupling of this ring with the diazo-reagent. Experiments are in progress with the

object of elucidating this problem. In either case, whether the loss of carnosine is due to a synthesis or to a breaking up of the base, it will be of interest to know the fate of the β-alanine portion of the molecule, as this is the only known β-amino acid in the animal organism.

The stepped nature of the curves of loss of carnosine is peculiar and totally unlike any enzyme curve. It may indicate that not one but two agents are active in the change, and that they act in an analogous way to enzymes and co-enzymes. On the other hand the catalyst may be a single substance and the reaction take place in two stages separated by an inactive period. Neither of these explanations can account for the initial delay shown when using washed meat as a source of catalyst, since after this delay the three-stepped curve appears.

The catalyst is present in ox, rat, and cod muscle and therefore is probably found in vertebrate skeletal muscle generally, but experiments with lobster and oyster muscle indicate its absence from invertebrates. The livers of the rat and ox also contain this catalyst, which however is absent from frozen ox-liver. This indicates another connection between hepatic and muscular activity already seen in glycogen metabolism and also with urea, creatine and creatinine.

The catalyst is not present in all cells since experiments have proved it to be absent from the kidney of the rat, ox, and sheep. The existence of this catalyst may account for the non-appearance of ingested carnosine in the urine.

Earlier experiments in which carnosine was shown to be absent from the striped muscles of the white fishes and the finches appeared to suggest that carnosine is a merely accidental substance, somewhat arbitrary in its presence or absence. But the experiments detailed above, particularly those that show the presence of a carnosine-removing catalyst in the muscle of white fish, suggest that it is an intermediary product of metabolism and that its appearance and its percentage in muscle are determined by the rate at which it is formed and the rate at which it is removed in the different types of muscle.

The expenses of this research were defrayed by a grant from the Medical Research Council.

Thanks are due to Professor V. H. Mottram for the interest shown by him in the work and for his helpful criticism.

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LXXXIV. ON THE VITAMIN D.

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(*Received October 25th, 1922.*)

The well-known fact, first described by Wildiers, that yeast cells when in low concentration fail either to ferment sugar or to grow, but may be induced to do so by the addition of "bios," is as yet imperfectly understood. The need of yeast cells for "bios" depends clearly on dilution; for yeast, when in adequate initial concentration, is able to synthesise this substance indefinitely from the ingredients of a simple medium. It is an organic substance, soluble in water or alcohol, dialysable, and thermostable; it appears therefore to belong to the group of the vitamins. It seems, however, to be more thermostable than the vitamin which promotes the growth of rats [Souza and McCollum, 1920]; it is more thermostable also than the co-enzyme of zymase [Tholin, 1921]. The capacity for activating yeast in this manner has been used nevertheless by Williams [1919] and others [see Sherman, 1921], as a measure of the richness of any material in the vitamin B; and there is no doubt that in many cases, in yeast itself for instance, this vitamin and "bios" are closely associated.

Now the comparative value of the various organs of animals, for preventing the onset of polyneuritis in pigeons fed on polished rice, has been determined by Cooper [1914], who places them in the following order: liver, heart, cerebrum, cerebellum, muscle; 0·5 g. of yeast being equivalent for this purpose to 0·9 g. of liver, 1·2 g. of brain, or 5·0 g. of muscle (dried weights in each case). In respect of promoting the growth of young rats, Osborne and Mendel [1918] place the tissues in a similar order. The distribution of "bios" among the organs of animals, therefore, seemed of interest as a test of the closeness of association between it and this vitamin; and a comparison in this respect between the organs of normal animals and those of animals suffering from vitamin-B deficiency, as an indication how far this deficiency can be attributed to a shortage of "bios." Such an investigation forms the subject of the experiments to be described.

Method.

Two methods have been adopted for measuring "bios," or the substance which activates yeast; the one depends upon the rate of multiplication of yeast cells, the other upon the rate of fermentation, as indicated by the evolution of CO_2. The majority of workers have preferred to study growth,

as being a less erratic phenomenon than that of CO_2 production. The number and complexity of the factors which influence growth, however, are likely to be no less great than those which influence fermentation; and the fermentation method has been used in the present experiments, since it has the advantages of convenience and speed, and allows therefore many estimations to be made and an average drawn.

The medium employed has been Nägeli's solution, omitting ammonium nitrate. Ammonium nitrate of course is necessary for the growth of yeast, and leads also to more rapid fermentation; but where the rate of fermentation by a given quantity of yeast is alone in question, a factor which only promotes growth is a source of error; and results without it, while less sensitive, seemed likely to be more accurate than in its presence. The composition of the medium used, then, was:

Cane sugar	10 g.
Potassium biphosphate ...	0·5 g.
Magnesium sulphate	0·25 g.
Calcium phosphate	0·05 g.
Distilled water to	100 cc.

To this medium is added 0·01 % of fresh brewer's yeast (1 cc. of a 1 % suspension to 100 cc.), a quantity in itself insufficient to produce any fermentation whatever in 24 hours: this constitutes the standard yeast-Nägeli medium. To 10 cc. in each of a series of tubes is added a graduated quantity of a watery extract of the substance under investigation. The mixtures are transferred with a serum-syringe to a series of 3 cc. glass ampoules, such as are used for vaccines, two ampoules from each tube. The experiments are performed in duplicate, so that four determinations are made for each concentration of the activating substance. The ampoules are then inverted over a receptacle, and incubated at 33° for 24 hours. As fermentation occurs, fluid is displaced, and drips out of the neck of the ampoule into the receptacle. Each ampoule is weighed empty (A), full (B), and at the end of the experiment (C). The difference, $B-C$, represents the cc. of CO_2 produced in $B-A$ cc. of solution; from which the percentage CO_2 formation may be calculated.

Results found to be most reliable are obtained when the fermentation amounts to about 5–10 %; the yeast tends to adhere to the glass as the fluid recedes, vitiating results when larger amounts of fermentation have occurred. And the progressive emptying of the ampoule constantly diminishes the quantity of fluid in which the recorded fermentation is taking place. Other influences, moreover, which may affect the rate of fermentation, such as the presence of amino-acid or of phosphate, cause an increasingly greater percentage error. The suggestion has indeed been made that such influences completely vitiate the test. At these concentrations of yeast, however, the error that they produce is not large. Table I shows the fermentation produced in the medium, in the absence of a specific activating substance, by various influences of this kind.

Table I. *Showing absence of fermentation in standard yeast-Nägeli medium, in absence of specific activating substance.*

Added to 10 cc. yeast-Nägeli medium ...	·05	·1	·15	·2 cc.
	Percentage fermentation			
Sodium phosphate 2 %	0	0	1	1
Commercial glucose 2 %	1	1	1	1
Dried blood 2 % extract	1	1	1	2

Table II shows, further, that when an extract of dried yeast, which is an efficient activating substance, is added to the medium, the further addition of phosphate makes very little difference.

Table II. *Showing absence of effect of sodium phosphate on fermentation produced in activated yeast-Nägeli medium.*

10 cc. yeast-Nägeli medium, with 0·075 cc. of 2 % extract of dried yeast	Fermentation
Without sodium phosphate	4·5
With sodium phosphate	4·25

A more searching test, however, as to whether the activation of minimal quantities of yeast is or is not the effect of a single substance, and whether the fermentation thereby produced is or is not a satisfactory measure of such substance, is afforded by a comparison of the fermentation curves obtained when the activating substance is derived from various sources. The following have been used for this purpose: cow's milk, a 10 % solution of "Glaxo," a 2 % extract of the desiccated spleen of the calf (Armour), and a 2 % extract of dried yeast. Table III represents the average of a large number of determinations of the fermentation produced in the standard medium, after addition of these four substances in increasing quantity.

Table III. *Average fermentation obtained in standard yeast-Nägeli medium, after addition of increasing amounts of various activating substances.*

Amount added to 10 cc. yeast-Nägeli medium ...	·05	·075	·1	·125	·15	·175	·2	·225	·25	·3 cc.
Substance added	Percentage fermentation in 24 hours									
Cow's milk	2·5	4·6	8·6	11·8	16·5	16·9	23·4	—	—	—
10 % Glaxo	—	—	4	—	7	—	8·5	—	15	24
2 % dried yeast ...	1·5	3·3	7·7	11·6	16·2	24·2	—	—	—	—
2 % desiccated spleen ...	—	2·5	4·4	6·5	12	14·5	18·1	21·5	—	—

It is clear from this table that while the activating power of cow's milk is almost exactly the same as that of 2 % yeast extract, it is considerably greater than that of either 10 % Glaxo or 2 % spleen. If, however, the activating powers be calculated from the above figures, of 17 % Glaxo and of 2·6 % spleen respectively, and the results plotted on a curve, it is found that such fermentation curves, for the four activating substances used, almost exactly coincide (Fig. 1). This seems to indicate that the activating substance is a single and definite entity, and that the method is a fair one for estimating its quantity in any material.

In the following experiments the curve shown in Fig. 1 has been taken as a standard, indicating the fermentation produced by a 2 % extract of dried yeast. The organs of various animals have been air-dried at 33°, and powdered, and their activating power determined in 2 % watery extract. By comparison with Fig. 1 (or Table III) it is possible to say how much dried yeast is the equivalent in activating power of unit weight of the material investigated. This, for convenience, I have called the "yeast-equivalent" of the substance. From Table III, for instance, it will be seen that the yeast-equivalent of this preparation of desiccated spleen is 0·8.

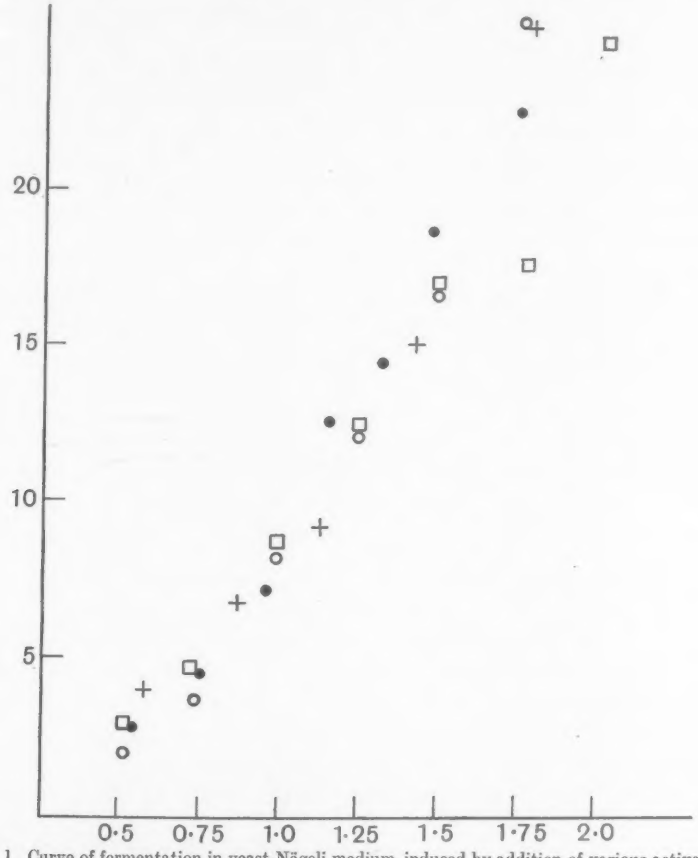

Fig. 1. Curve of fermentation in yeast-Nägeli medium, induced by addition of various activating substances.

Abscissae: Percentage addition to the medium of the various substances. Ordinates: Percentage fermentation in 24 hours at 33°. Crosses: 17 % Glaxo[1]. Squares: Cow's milk. Hollow circles: 2 % dried yeast. Solid circles: 2·6 % desiccated spleen[1].

[1] Calculated from observations with 10 % Glaxo and 2 % spleen respectively.

Results.

(1) *Pigeons.* The organs were taken and dried under the same conditions from a series of birds, being (1) normal pigeons, and (2) pigeons which on an exclusive diet of polished rice had developed avian polyneuritis, and had either died from this condition, or had been killed at the point of death. It was thought that if the substance which activates yeast were the same as that whose absence causes polyneuritis, the organs of polyneuritic birds should show a deficiency of this substance; and this deficiency should be most marked in the brain, where the symptoms are so nearly exclusively localised. Funk [1912], as is well known, has shown a deficiency in the N- and P-content of the brains of birds in this condition.

Because the symptoms of avian polyneuritis are markedly cerebellar in type, the cerebral hemispheres and cerebellum were dried and estimated separately. In order to illustrate the degree of consistency of the results, the figures obtained in the case of the brain (cerebral hemispheres) are given in detail in Table IV. Table V is a summary of results from all organs, the average alone being given in each case, together with the calculated "yeast-equivalent."

Table IV. *Details of experiments showing percentage fermentation obtained in yeast-Nägeli medium by addition of* 2 % *extracts of dried cerebral hemispheres of pigeons.*

Pigeon	1	2	3	4	Av.	1	2	3	4	Av.
I. Polyneuritic										
P	2·5	6	2·5	7·5	4·9	11	11	11·5	9	10·5
Q	4·5	3·5	4	4·5	4·1	9	7	8	4·5	7·1
O	3	3	1·5	1·5	2·2	5	5	8·5	6	6·1
Y	8	4	3·5	5·5	5·2	8	9	10	10	9·2
Z	4	8	4	5	5·2	9	11	10	12	10·5
B	3	2	1·5	3·5	2·5	6	9	10	6	7·7
X	3·5	3	4·5	4	3·8	8	5	7·5	4	6·1
Average	4·1	4·2	3·1	4·9	4·0	8	8·1	9·4	7·4	8·2
II. Normal										
D	5	3	5·5	4	4·4	8	11·5	11	6	9·1
H	5	3	5	5	4·5	9	12	10	5	9·0
A	4	5	6	4	4·7	11	10	9	11	10·2
Average	4·7	3·7	5·5	4·3	4·5	9·3	11·2	10	7·3	9·5

Tables IV and V show, firstly, that the power to activate yeast is approximately the same in all the organs of pigeons that were examined, and that this power is not very much less than that of dried yeast itself: the liver, which is slightly more active than other organs, being scarcely at all inferior to yeast. This offers a striking contrast with the figures of Cooper, already referred to, showing the relative efficiency of the organs in preventing the development of polyneuritis.

So marked a contrast seems to show quite clearly, not only that the yeast-activating substance is not identical with the antineuritic vitamin, but that the two are not associated together in constant relative proportions.

Table V. *Showing average fermentation produced in standard yeast-Nägeli medium by addition of 2 % extracts of dried organs of pigeons, and "yeast-equivalents" calculated therefrom.*

Organ ...	2 % cerebrum			2 % cerebellum			2 % liver			2 % kidney			2 % heart			2 % muscle		
Cc. added to 10 cc. yeast-Nägeli medium ...	·1	·15		·1	·15		·1	·15		·1	·15		·1	·15		·1	·15	
Pigeon	%	Ferm.	Yeast equiv.	%	Ferm.	Yeast equiv.	%	Ferm.	Yeast equiv.	%	Ferm.	Yeast equiv.	%	Ferm.	Yeast equiv.	%	Ferm.	Yeast equiv.
I. Polyneuritic																		
P	5	10·5	·8	1·5	4	·4	9·5	14·5	1·0	7	12	·9	8	12	·95	5	11	·8
Q	4	7	·7	5	12	·85	4·5	12	·8	4	8	·75	5	9	·75	5·5	11	·85
O	2	6	·5	4	9	·75	8·5	14	1·0	4·5	10	·8	4	8	·75	4	6	·7
Y	5	9	·8	5·5	11	·85	7	14	·9	—		·85	—		—	—		—
Z	5	10·5	·8	—		—	8	9·5	·9	—		—	—		—	—		—
B	2·5	7·5	·6	—		—	8	12	·9	6	10·5	·85	6	8	·8	5·5	9·5	·8
X	4	6	·6	3·5	8	·7	6·5	8·5	·8	—		—	—		—	—		—
Average	4	8·2	·7	4	9	·75	7·5	12	·9	5·4	10	·8	5·7	9	·8	5	9·5	·8
II. Normal																		
D	4·5	9	·8	5	10	·8	8·5	25	1·1	4	8	·75	6·5	11	·85	4	5	·6
H	4·5	9	·8	4	8	·75	—		—	—		—	—		—	—		—
A	4·5	10	·8	—		—	5	7·5	·7	8	14	1·0	4·5	9	·8	5	8	·7
Average	4·5	9·3	·8	4·5	9	·8	6·7	16·3	·9	6	11	·85	5·5	10	·8	4·5	6·5	·7

The tables show, moreover, secondly, that the activating power of the organs of polyneuritic pigeons is only very slightly less than that of the normal organs. This fact serves to strengthen the conclusion that deficiency of the substance which activates yeast is not the deficiency which causes polyneuritis.

There might indeed be a possibility that the organs of polyneuritic animals, while retaining their percentage of this activating substance, suffered a loss in their total content, by diminution in their whole weight. However, as Abderhalden has shown [1921], such a diminution in the weight of individual organs in polyneuritis occurs only in the case of the liver and voluntary muscles, not in that of the brain. This observation was confirmed in the foregoing experiments, as is seen in Table VI.

Table VI. *Showing weights of normal and of polyneuritic pigeons, and of their organs.*

Pigeon	Total body weight	Weights of organs		
		Cerebrum	Cerebellum	Liver
I. Polyneuritic				
P	150	0·98	0·22	4·52
Q	165	1·05	0·20	7·78
O	145	1·13	0·22	3·15
Y	150	1·12	0·23	5·22
Z	165	1·01	0·26	6·10
B	224	1·11	0·27	8·67
X	199	0·94	0·27	5·85
Average	171	1·05	0·24	5·90
II. Normal				
D	270	1·08	0·28	6·39
H	—	1·03	0·25	—
A	323	1·15	0·24	6·86
Average	296	1·09	0·26	6·68

(2) *Rats.* It follows from the above experiments, that the yeast-activating substance cannot be identified with the antineuritic vitamin, its amount being very nearly as great in the organs of polyneuritic as in those of normal pigeons. Similar experiments were performed on rats, the organs of normal animals being compared with those of others, which had been either killed when moribund, or had died, as the result of a diet free from water-soluble vitamin; and with those of a third series which, as the result of such a diet, had ceased to grow for several weeks, but had been killed before any other symptoms had manifested themselves. The diet consisted of the usual mixture of purified caseinogen, starch, sugar, butter, and salts. Upon this diet growth ceased at once; but if the initial weight of the animals was not less than about 70 g., they remained active and in good condition for many weeks; eventually dying with or without irregular nervous symptoms. The course of this condition, as has often been observed, is in striking contrast with the regular and rapid onset and constant symptoms of polyneuritis in birds. Table VII gives the summary of the average results of experiments on the content of the organs of these rats in the substance which activates yeast.

It will be seen that in the case of normal rats, the "yeast-equivalents" approximate very closely to those found in pigeons; but that in the rats which had died as the result of deprivation of water-soluble vitamin, this "equivalent" has diminished considerably. The rats which were killed after a shorter period (six weeks) of vitamin starvation occupy in this respect an intermediate position.

Table VII. *Showing average percentage fermentation produced in standard yeast-Nägeli medium by addition of 2 % extracts of dried organs of rats, with "yeast-equivalents" calculated therefrom.*

Organ ... Cc. added to 10 cc. yeast-Nägeli medium	2 % brain				2 % liver				2 % kidney				2 % heart			
	·1	·15	·2		·1	·15	·2		·1	·15	·2		·1	·15	·2	
Rat number	% fermentation			Yeast equiv.	% fermentation			Yeast equiv.	% fermentation			Yeast equiv.	% fermentation			Yeast equiv.
I. Normal rats																
11	5·5	13	—	0·85	5·5	17	—	0·9	14	28·5	—	1·3	5	10	16	0·8
6	5	11	19·5	0·8	6	14	—	0·9	7	17	—	0·9	5·5	9	15	0·8
7	8	20	—	1·0	3·5	6	—	0·6	5·5	16	—	0·9	5	7	16	0·75
Average	6	15	—	0·9	5	12	—	0·8	9	20·5	—	1·0	5	9	16	0·75
II. Vitamin-free diet six weeks. No symptoms																
8	4·5	6	13	0·7	5	10	15	0·8	6	9	14	0·8	3·5	4·5	6	0·6
9	2	3	9	0·5	4	9	13	0·75	7·5	9	16	0·85	1·5	2·5	6	0·45
10	2	4	8	0·5	2·5	4	8	0·5	6	11	14	0·8	3	7	10	0·6
Average	3	4·3	10	0·6	4	8	12	0·7	6·5	9·7	15	0·8	2·7	4·7	7	0·5
III. Vitamin-free diet till death or moribund																
1	1	—	8·5	0·5	1·5	—	11	0·6	2·5	—	7·5	0·5	1	—	2·5	0·3
2	2	—	10·5	0·55	1	—	5	0·4	1	—	5	0·4	2	—	7·5	0·5
3	1·5	—	10	0·55	1·5	—	7·5	0·45	4	—	11	0·55	1	—	6	0·45
4	1·5	—	6·5	0·45	3	—	7·5	0·5	—	3	8	0·5	—	1	6	0·4
5	—	6	9	0·55	—	7	9	0·55	—	9·5	15·5	0·8	—	3	4·5	0·4
Average	1·5	6	9	0·55	1·5	—	8	0·55	2·5	6	9·5	0·6	1·5	2	5·3	0·4

There is therefore a considerable difference between the condition of these rats and that of polyneuritic pigeons. This is due, no doubt, to the long duration of the diet; for pigeons on a diet of polished rice are invariably dead from polyneuritis within 28 days. But it shows at any rate, that rats suffering from deficiency of water-soluble vitamin are depleted of a substance which polyneuritic pigeons still possess; since death occurs before this second deficiency is manifested.

These findings afford support to the conclusions of Funk and Dubin [1921], that there are two water-soluble vitamins present in yeast: it is the absence of one of these, vitamin B, which causes polyneuritis in birds; while the other, which these authors call vitamin D, is the "bios" of Wildiers, necessary to the activity of low concentrations of yeast cells; both of them are necessary for the proper growth of rats. The prevailing uncertainty as to the identity of the antineuritic and the growth-promoting vitamins is due, therefore, to the fact that the latter includes the antineuritic substance, but contains also another essential factor, the vitamin D.

So far as yeast is concerned, the "bios" effect indicates that the vitamin D alone is necessary for the activation of low concentrations of yeast cells, and leads not only to fermentation but also to growth. This must not be taken to imply, however, that fermentation and growth are identical phenomena; nor that vitamin D is the only requisite for the manifestation of either or both of them. The fact that vitamin B is present in yeast cells is a proof to the contrary; and other vitamins, it may be, are responsible for various functions in the life-history of the yeast-plant. The "bios" effect signifies merely that vitamin D is one of the substances necessary to the life of the cell, and that it differs from most other such substances in undergoing dilution by diffusion into the surrounding medium.

Summary.

(1) The substance which activates minimal concentrations of yeast is a definite chemical entity, though obtainable from many sources. It may be measured fairly satisfactorily by the fermentation to which it gives rise.

(2) It is present, in approximately the same amount, in the following organs, both of pigeons and rats: cerebrum, cerebellum, liver, kidney, heart, and voluntary muscle; its amount in any of these is about 80 % of its amount in dried yeast. This distribution is contrasted with that of the antineuritic vitamin.

(3) In the organs of pigeons rendered polyneuritic by a diet of polished rice, it is present in the same amount as in those of normal pigeons.

(4) In the organs of rats fed on a diet purified of water-soluble vitamin, its amount progressively diminishes, and when death occurs is only about one-half the normal.

(5) For these reasons this activating substance cannot be identified with

the antineuritic vitamin B. The deprivation of water-soluble vitamin from rats is held to involve a double deficiency; a deficiency of vitamin B in the first place, and of the yeast-activating substance in the second. This is the substance which Wildiers called "bios," and Funk the vitamin D.

The author wishes to express his cordial thanks and obligation to Dr J. A. Gunn, Professor of Pharmacology in the University of Oxford, for facilities to carry out the above work in his laboratory.

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LXXXV. A NEW PHOSPHORIC ESTER PRODUCED BY THE ACTION OF YEAST JUICE ON HEXOSES.

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(*Received October 30th, 1922.*)

The effect of sodium phosphate in increasing the fermentative power of yeast juice was first observed by Wroblewski [1901] and again by Buchner [Buchner, E. and H. and Hahn 1903] by whom it was attributed to the alkalinity of the salt. That such was not the true explanation followed from the work of Harden and Young [1905; 1906, 1 and 2; 1908, 1 and 2; 1909; 1910, 1 and 2; 1911] whose extended investigations revealed the important rôle played by phosphates in alcoholic fermentation. It was shown by these authors that when a suitable amount of a soluble phosphate is added to a fermenting mixture of yeast juice and glucose, fructose or mannose, the rate of fermentation rapidly increases but after a short period again falls to a constant rate, which is only slightly greater than that of the original yeast juice and sugar. During this period of increased fermentative activity the total evolution of carbon dioxide is increased by an amount which is equivalent, molecule per molecule, to the phosphate added, while the latter undergoes a transformation into a form that is no longer precipitable by magnesium citrate mixture.

From such solutions Young [1907; 1909] succeeded in isolating an ester of phosphoric acid by precipitation in the form of its insoluble lead salt. He showed [1909; 1911] that this compound possessed the constitution of a hexosediphosphoric acid, $C_6H_{10}O_4(PO_4H_2)_2$, and that the same substance was formed from glucose, fructose or mannose when fermented with yeast juice or zymin in the presence of phosphate. The acid was slightly dextrorotatory ($[\alpha]_D = + 3{\cdot}2°$) and on hydrolysis yielded phosphoric acid and a laevorotatory reducing substance from which fructose was isolated, although Young was not satisfied that the latter was the only sugar produced. A number of salts and derivatives were described, including the phenylhydrazine salt of a phenylhydrazone in which both phosphoric acid groups were retained, and an osazone in the formation of which one molecule of phosphoric acid had been split off. The evidence brought forward by Young was, however, insufficient to decide fully the constitution of the ester.

About the same time this compound was also discovered by Ivanov [1905; 1907; 1909], who attributed to it the constitution of a triosemonophosphoric ester, and at a somewhat later date was examined by Lebedev [1909; 1910] who at first supposed it to be a hexosemonophosphoric ester but afterwards [1911] came to the same conclusion as Young on this point.

During the course of some experiments carried out in 1913 by the author in conjunction with Professor A. Harden indications were noted that hexosediphosphoric acid is not the only compound of this type produced during the fermentation of sugar by yeast juice in presence of phosphate. It was observed that during the preparation of hexosediphosphoric acid from fructose and after the precipitation of its lead salt by the addition of lead acetate, the filtrate always contained very appreciable amounts of phosphorus in organic combination, and that a further precipitate containing most of this phosphorus was thrown down on the addition of basic lead acetate. On examining the acid solutions obtained by decomposing such basic lead precipitates with hydrogen sulphide it was found that the ratio of the reducing power, as determined by Bertrand's method, to the phosphorus content was much higher than that given by solutions of hexosediphosphoric acid, while polarimetric observations gave evidence of the presence of a substance much more strongly dextrorotatory than the latter.

The barium salt of this new acid was prepared, and proved to be readily soluble in both hot and cold water, differing in this respect markedly from barium hexosediphosphate. The salt was amorphous and was obviously contaminated with substances derived either from the yeast juice or formed during the fermentation of the fructose, but the results of analyses appeared to indicate that the compound was the barium salt of a hexosemonophosphoric acid, $C_6H_{11}O_5PO_4Ba$. These facts were published in a preliminary note [Harden and Robison, 1914] and the investigation of the compound was continued. Its purification, however, proved unexpectedly difficult and was not completed when the work was interrupted by the war. It was resumed in 1919 but was again held up by the difficulty experienced in obtaining yeast juice of reasonable fermentative power. The variability in the activity of juice prepared from the same type of yeast and apparently under similar conditions has been frequently observed, but no explanation could be found for the long run of inactive or feebly active juices obtained during 1919 unless it were the disheartening effect of the war beer on the yeast. That difficulty no longer exists and improved methods for the isolation and purification of the new compound have been worked out. Sufficient evidence has now been obtained to confirm the opinion already stated as to its constitution. It is, however, not identical with the hexosemonophosphoric acid prepared by Neuberg [1918], by partial hydrolysis from hexosediphosphoric acid. A specimen of barium hexosemonophosphate prepared according to the method described by Neuberg was found to be optically almost inactive but to yield a strongly laevorotatory product on further hydrolysis. The compound iso-

lated from the products of fermentation is, on the other hand, strongly dextrorotatory, and on hydrolysis yields a dextrorotatory reducing substance from which glucosazone has been obtained. The ratio of the rotatory power of this hydrolysis product to its reducing power as estimated by Bertrand's method is, however, much lower than that given by solutions of pure glucose. In this and in other respects the behaviour of the ester suggests that it may possibly be a mixture of isomeric hexosemonophosphoric acids, while the substance described by Neuberg may represent one or more other isomers. The isomerism may be due to the hexose molecule itself or to the position of the phosphoric acid radicle.

So far, only one salt of hexosemonophosphoric acid, that of brucine, has been obtained in a crystalline condition and the hope that this salt might be used to effect the purification of the acid, and its possible resolution into two or more isomers was for long disappointed owing to the difficulty with which crystallisation was effected. Within the last few weeks, however, more success has been obtained and it is hoped that the question may yet be solved in this way.

With phenylhydrazine, hexosemonophosphoric acid yields the phenylhydrazine salt of an osazone in which the phosphoric acid radicle is retained. This compound is not identical with the osazone of the same empirical formula prepared by Young, Ivanov and Lebedev from hexosediphosphoric acid.

Hexosemonophosphoric acid is only very slowly hydrolysed by boiling with mineral acids and a considerable portion of the sugar produced is destroyed during the operation. It is also hydrolysed by emulsin, yielding free phosphoric acid and a dextrorotatory reducing substance. Emulsin likewise hydrolyses hexosediphosphoric acid but, as with acid hydrolysis, the reducing substance produced is strongly laevorotatory. It is therefore improbable that hexosemonophosphoric acid is formed during the fermentation by partial hydrolysis—enzymic or otherwise—of the diphosphoric ester.

The behaviour of hexosemonophosphate towards yeast, yeast juice and zymin is being studied in conjunction with Dr Harden and is yielding interesting results that are, however, difficult to interpret. The alkali salts are readily fermented by yeast juice and by zymin and the initial rate is relatively high, even approximating to the "phosphate rate" for glucose, but rapidly falls to a lower level, which nevertheless is decidedly higher than that for hexosediphosphate.

The same compound is apparently formed during the fermentation of either fructose or glucose in presence of phosphate. It is true that some differences were observed in the specific rotations of the various preparations, but such differences were not greater between preparations made from glucose and those from fructose than between different preparations made from the same sugar. This may perhaps be taken as further evidence that all specimens were mixtures of isomeric compounds in somewhat varying proportions.

Owing to the many processes employed in the different attempts to obtain

the ester in a pure condition, and the fact that even the best of these methods involves some loss of the substance, the record of yields obtained does not at present give very trustworthy evidence as to the most favourable conditions for the production of the monophosphoric ester or for determining the relative amounts of this and the diphosphoric ester that are formed during fermentation.

Calculations based on the values of the ratio

$$\frac{\text{Reducing power (as glucose)}}{\text{Phosphorus}}$$

as determined for the acid solutions derived from both normal and basic lead precipitates would appear to indicate the presence of a much higher proportion of the monophosphoric ester than has ever been actually isolated. It is just possible that in spite of the thorough washing to which they were subjected, these precipitates still held some adsorbed sugar which would affect the above ratio. The possibility of other reducing substances being present must also be considered.

The best yields of the hexosemonophosphoric acid actually obtained from fructose and from glucose correspond with only 15 % and 7 % respectively of the sodium phosphate added during the fermentation. These figures are based on the weight of barium salt of at least 90 % purity; the total amounts formed would certainly be higher and might well correspond with double these percentages.

The question as to what rôle, if any, is played by hexosemonophosphoric acid in alcoholic fermentation must be left unanswered until more information is gained on the various points indicated above.

Experimental.

A typical series of values of the ratios

$$\frac{\text{Reducing power (as glucose)}}{\text{Phosphorus}} \text{ and } \frac{\text{Rotation in 4 dm. tube}}{\text{Reducing power (as glucose) in 100 cc.}}$$

as determined for the acid solutions derived from normal and basic lead precipitates, is given below. They are taken from one of the first experiments, which led to the discovery of the monophosphoric ester, and are quoted because of their bearing on the relative amounts of the two esters formed during the fermentation.

The reducing power was estimated by Bertrand's method and calculated as glucose; the phosphorus was estimated by Neumann's method.

Fermentation: 1220 cc. juice from 4 kilos of fresh pressed brewery yeast was allowed to ferment 122 g. fructose, 131 g. (0·4 Mol.) of $Na_2HPO_4 12H_2O$ being added gradually, as indicated by the rate of evolution of CO_2, during $1\frac{1}{2}$ hours. Fermentation was stopped and protein coagulated by blowing steam through the solution. After neutralisation of the filtered solution with caustic soda and removal of free phosphate by precipitation with magnesium acetate, a slight excess of lead acetate was added to the filtrate. The precipitate was

removed by centrifuging and washed nine times with water. The filtrate and washings were treated with basic lead acetate and the precipitate again separated by centrifuging and thoroughly washed. These precipitates were decomposed by hydrogen sulphide and the excess of the latter removed by means of a current of air. The acid solutions were each neutralised to phenolphthalein by the addition of caustic soda and were again treated successively with normal and with basic lead acetate, any traces of free phosphate being first removed. These operations were repeated as shewn below (Table I).

The acid solutions derived from the various lead precipitates gave the following ratios:

Table I.

No. of sol.	Description of precipitate from which solution was derived	Reducing power as glucose P	Rotation in 4 dm. tube / Reducing power as glucose in 100 cc.	$[\alpha]_D$ of acid calculated on P-content
1	First precipitate by normal lead acetate (crude lead hexosediphosphate)	2·50	—	—
2	Solution 1 precipitated a second time by normal lead acetate	2·25	—	—
3	Solution 2 precipitated a third time by normal lead acetate. Precipitations carried out in two fractions: 1st fraction	1·50	—	—
4 *a*	Solution 3 precipitated a fourth and fifth time with normal lead acetate, the fifth time in two fractions: 1st fraction	1·21	0·647	Calculated for $C_6H_{10}O_4(PO_4H_2)_2$ +3·56°
4 *b*	2nd „	1·67	—	—
5	Solution 4 *a* precipitated a sixth time with normal lead acetate (pure lead hexosediphosphate)	1·16	0·623	+3·26°*
6	First precipitate by basic lead acetate after removal of first normal lead precipitate	5·50	—	—
7 *a*	Solution 6 precipitated a second time with normal then with basic lead acetate: Normal lead precipitate ...	—	—	—
7 *b*	Basic „ „ ...	4·24	—	—
8 *a*	Solution 7 *b* precipitated a third time with normal then with basic lead acetate: Normal lead precipitate ...	3·96	1·60	Calculated for $C_6H_{11}O_5(PO_4H_2)$ +19·0°
8 *b*	Basic „ „ ...	4·36	2·40	+31·2°
9 *a*	Solution 8 *b* precipitated a fourth time with normal then with basic lead acetate: Normal lead precipitate ...	4·13	2·18	+25·8°†
9 *b*	Basic lead precipitate Amount too small for determination of ratios	—	—	—

* $[\alpha]_D$ of hexosediphosphoric acid = +3·2° (Young).

† $[\alpha]_D$ of hexosemonophosphoric acid = +25·0° (p. 817).

Solutions 9 *a* and 9 *b* were each treated with baryta until neutral to phenolphthalein and the barium salts precipitated by the addition of an equal

volume of alcohol. They were purified by redissolving in water and reprecipitating with alcohol and were then dried at 100° to constant weight and analysed:

Barium salt from solution 9 *a*. Ba = 34·33 %; P = 7·07 %.
" " " " 9 *b*. Ba = 34·86 %; P = 7·22 %.
Calculated for $C_6H_{11}O_5PO_4Ba$. Ba = 34·75 %; P = 7·85 %.

It seemed probable therefore that the chief constituent of the basic lead precipitates was the salt of a hexosemonophosphoric acid, but that this was contaminated with salts of other acids containing less phosphorus or none at all. Whether the differences in the ratios for solutions Nos. 6 to 9 are due entirely to phosphorus-free compounds, or whether they indicate the presence of yet another phosphoric ester, *e.g.* of a disaccharide, is impossible at present to say.

In the case of the solutions derived from the normal lead precipitates a steady decrease in the ratio Reducing power/P occurs as the purification proceeds. If the final ratio of 1·16[1] be accepted as correct for pure hexosediphosphoric acid, then it is obvious that solution 1 must have contained a considerable proportion of some other reducing substance. The presence of sugar would seem to be precluded by the thorough washing of the precipitate. That the precipitate contained some hexosemonophosphate is certain since, although this ester gives in concentrated neutral solutions no precipitate with normal lead acetate, a copious precipitate, probably of basic lead salt, is formed in dilute solutions.

If solution No. 1 contained no reducing substance or phosphorus compound other than the two hexosephosphoric acids, then the ratio 2·50 would indicate that nearly 50 % of the phosphorus was present in the form of the mono-ester, in addition to the amount remaining in the filtrate and precipitated by basic lead acetate. As already stated, the yields of hexosemonophosphoric acid actually isolated in the form of its barium salt have never reached this proportion and have usually been much lower.

The purification of the ester proved a problem of considerable difficulty and much time was spent in solving it. Attempts to prepare salts in a crystalline condition were unsuccessful except in the case of the brucine salt, and fractional precipitation of the metallic salts by alcohol effected only slight improvement in the analytical figures. Fractional precipitation of the syrupy acid from its alcoholic solution by ether, was also of little use. Repeated extraction of the aqueous solution of the acid with ether or ethyl acetate did remove a small proportion of crystalline substance which proved to be succinic acid, but the phosphorus content of the barium salt was thereby only slightly raised. Further fractional precipitation by lead acetate, normal and basic, gave a purer product but involved too rapid diminution in the amount of

[1] The values for this ratio calculated from Young's data lie between 2·1 and 2·3. They are, however, not comparable with those shown above as the reducing power was determined by a different method(Pavy).

substance to be practicable. The greatest improvement was effected by the use of mercuric acetate which precipitates a considerable proportion of the impurities and by combination of this with other processes, a product giving satisfactory analytical figures was at length obtained.

Preparation and purification of the hexosephosphoric esters.

The methods employed in the preparation of the esters have been considerably modified as experience suggested. Heating the fermentation mixture to destroy the enzymes and coagulate the protein was discontinued on account of the risk of causing partial hydrolysis of the diphosphoric acid. Preliminary separation of the two esters by means of their lead salts was given up owing to the large proportion of the monophosphoric acid brought down in such dilute solutions by lead acetate. The method now used is as follows:

Juice prepared from fresh pressed English mild ale yeast is mixed with 10 % of its weight of fructose or glucose and warmed to 26° in a water-bath. Fermentation having commenced a 20 % aqueous solution of $Na_2HPO_412H_2O$ is added from time to time in such quantity as to produce the maximum rate of evolution of carbon dioxide. The addition of phosphate solution is best regulated by means of a guide experiment carried out with 25 cc. juice in the apparatus described by Harden, Thompson and Young [1910] (v. "Alcoholic Fermentation" by A. Harden, 1923 edition, p. 28). When the total volume of phosphate solution added exceeds half the original volume of juice a further quantity of sugar should be dissolved in the fermentation mixture. When the further addition of phosphate no longer causes any considerable rise in the rate of evolution of carbon dioxide, solid barium acetate in amount equal to the total weight of crystalline sodium phosphate that has been added, is dissolved in the reaction mixture which is then rendered just alkaline to phenolphthalein with baryta, and treated with an equal volume of alcohol. The barium salts of the two hexosephosphoric acids and of any excess of free phosphoric acid are thus precipitated together with the protein of the yeast juice. The precipitate is filtered off on a large Buchner funnel, and thoroughly washed with 70 % alcohol. It is then treated with boiling absolute alcohol and allowed to remain in contact with the alcohol over night. In this way the protein present is denaturated and rendered insoluble. The crude barium salts are then dried in an evacuated desiccator over sulphuric acid or more quickly by exposure to a current of warm air. They are next thoroughly ground up with 10 parts of cold water which dissolves the barium hexosemonophosphate, but scarcely any of the hexosediphosphate. The residue is washed twice with small quantities of water (being preferably removed from the Buchner funnel and again ground up) and is then extracted with 200 parts of water which dissolves the barium hexosediphosphate, leaving behind the insoluble barium phosphate etc. After removal of any traces of mineral phosphate by precipitation with magnesium acetate, the hexosediphosphoric acid is precipitated from the filtered solution in the form of its lead salt by

the addition of lead acetate. The precipitate is filtered or centrifuged off and washed. It is then suspended in water, decomposed by a current of sulphuretted hydrogen, the clear filtrate is freed from sulphuretted hydrogen by a current of air and finally neutralised to phenolphthalein with caustic soda. The precipitation of the lead salt is repeated several times [v. Young, 1909].

The first aqueous extract of the crude barium salts contains the barium hexosemonophosphate together with a little hexosediphosphate and other barium salts of acids derived from the yeast juice or formed during the fermentation. It is treated with basic lead acetate and the insoluble basic lead salt is filtered and washed. It is then decomposed with sulphuretted hydrogen as described above and the acid solution neutralised to phenolphthalein with a hot saturated solution of baryta. The solution is filtered and the precipitation with basic lead acetate repeated, after which the barium salt is again formed and is precipitated from its solution by alcohol, filtered off, washed with absolute alcohol and dried in an evacuated desiccator over sulphuric acid. It is dissolved in 10 parts of water to which 10 % of alcohol is finally added. The filtered solution is treated with mercuric acetate so long as this causes any precipitate to form, and is allowed to stand for some hours before filtering. The basic lead salt is then reprecipitated from the clear filtrate and once more converted into the barium salt, which is finally purified by repeatedly dissolving it in 10 % alcohol, filtering and reprecipitating with an equal volume of alcohol. In presence of a small proportion of water the salt readily forms a syrup, and it should therefore be washed several times with absolute alcohol and rapidly dried in an evacuated desiccator. If the results of analysis are not satisfactory the purification by mercuric acetate etc. is repeated. The mercury precipitate and a sparingly soluble barium salt, which is obtained in very small amounts during the final stage of the purification, both contain phosphorus and are being submitted to further investigation.

Hexosemonophosphoric acid and its salts.

Barium hexosemonophosphate, prepared by the above method, is a colourless, amorphous substance very readily soluble in hot and cold water, but insoluble in organic solvents. Dried over sulphuric acid it still retains about 4·5 % moisture which is given up at 100°, the salt at the same time taking on a brownish tint. The percentage of water corresponds approximately with one molecule (calculated for $C_6H_{11}O_5PO_4BaH_2O$, $H_2O = 4{\cdot}36$ %) but is not constant. The anhydrous salt takes up about the same amount of moisture with great avidity at ordinary temperatures even when kept in a closed vessel over calcium chloride.

For analysis the salt was dried to constant weight in an evacuated vessel (2 mm. pressure) at 78° over phosphorus pentoxide, only slight discoloration taking place under these conditions. Table II gives the results of analyses of specimens prepared from glucose and fructose, together with the specific

rotation of the barium salt and of the acid prepared from it by the addition of the exact amount of sulphuric acid.

The results obtained with a specimen of barium hexosemonophosphate prepared by hydrolysis of hexosediphosphoric acid (Neuberg's method) are also shown in the same table for purposes of comparison.

Table II.

No.	Sugar from which salt was prepared	Ba	P	C	H	$[\alpha]_D^{20}$ of salt	$[\alpha]_D^{20}$ of acid
1	Fructose	35·35	7·61	—	—	—	—
2	,,	34·89	7·86	—	—	+12·5° (c=3·9 %)	+24·9° (c=2·1 %)
3	Glucose	35·12	7·60	—	—	—	—
4	,,	35·05	7·66	—	—	+14·4° (c=14 %)	—
5	,,	34·82	7·45	—	—	+12·7° (c=17 %)	—
6	,,	34·83	7·73	—	—	+15·6° (c=11·2 %)	+28·8° (c=2·7 %)
7	,,	34·87 35·09	7·82 7·85	18·37 18·16	2·93 2·99	+12·2° (c=3·7 %)	+25·0° (c=3 %)
Calculated for $C_6H_{11}O_5PO_4Ba$		34·75	7·85	18·20	2·81	—	—
8	Hexosemonophosphoric acid obtained by partial hydrolysis of hexosediphosphoric acid (Neuberg's method)	33·26	7·58	—	—	+0·38° (c=5·2 %)	+1·49° (c=3·3 %)
Calculated for $C_6H_{11}O_5PO_4BaH_2O$		33·22	7·51	—	—	—	—

Molecular weight determination by the cryoscopic method yielded the following results:

1. 0·0849 barium salt in 13·17 g. water gave $\Delta = 0·062°$, whence apparent Mol. weight = 198.

2. 0·3037 barium salt in 13·17 g. water gave $\Delta = 0·192°$, whence apparent Mol. weight = 228.

$C_6H_{11}O_5PO_4Ba$ requires Mol. weight = 395·5; if 100 % dissociated, apparent Mol. weight = 197·8.

$C_{12}H_{20}O_9(PO_4Ba)_2H_2O$ requires Mol. weight = 791; if 100 % dissociated, apparent Mol. weight = 263·7.

The experimental results are therefore consistent with the first formula but not with the second.

The brucine salt was prepared by dissolving the calculated amount of the base in an aqueous solution of hexosemonophosphoric acid prepared from the barium salt. On evaporation of the solution at a low temperature a crystalline residue was obtained. This was very soluble in water, moderately soluble in warm methyl alcohol but sparingly soluble in ethyl alcohol. Recrystallisation was not readily effected but on treating the concentrated aqueous solution with acetone and cooling to 0°, a semi-crystalline precipitate was obtained. After twice repeating this operation the salt was dried to constant weight at 78° and 2 mm. pressure over phosphorus pentoxide, no discoloration taking place.

Phosphorus estimated by Neumann's method: found P = 2·73 %.

Calculated for $C_6H_{11}O_5PO_4H_2(C_{23}H_{26}N_2O_4)_2$; P = 2·96 %.

Specific rotation of salt in water (conc. = 6·6 %); $[\alpha]_D^{19} = -23·4°$.

The investigation of the brucine salt is being continued.

The normal lead and copper salts are also soluble in concentrated solutions but on diluting these, precipitates, presumably of the basic salts, are formed. Hexosemonophosphoric acid itself was obtained as a colourless, very viscous liquid by evaporating its aqueous solution *in vacuo*. It is moderately soluble in alcohol but very sparingly soluble in ether or acetone. Whether prepared from fructose or glucose it reduces Fehling's solution and gives a red coloration when heated with resorcinol and hydrochloric acid (Selivanov's reaction), though the colour is not so intense as that given by the equivalent amount of fructose. With water it yields a strongly acid solution which can be titrated sharply with alkalis (two equivalents) using phenolphthalein as indicator. All attempts to obtain the acid in crystalline condition have failed.

Osazone of hexosemonophosphoric acid.

Attempts to prepare a phenylhydrazone of hexosemonophosphoric acid were not successful but on warming the solution with excess of the base, a crystalline derivative was obtained. 10 g. barium hexosemonophosphate, prepared from fructose, were treated with the exact amount of sulphuric acid and the barium sulphate removed by filtration. The clear solution was mixed with 15 g. phenylhydrazine dissolved in acetic acid, and was heated on the water-bath during 30 minutes. It was then cooled to 0° and the yellow precipitate filtered off, washed with chloroform, and dried.

Weight of product = 8 g. M. pt. 135°–137°.

Recrystallisation was effected by dissolving the compound in boiling ethyl alcohol, adding an equal volume of hot chloroform and cooling to 0°. Short, pale yellow needles were formed. M. pt. 139° with decomposition. Moderately soluble in methyl alcohol, less soluble in ethyl alcohol, still less in chloroform and insoluble in light petroleum. Very sparingly soluble in water but readily soluble in dilute sodium hydroxide.

Analysis of substance dried at 66° *in vacuo* over phosphorus pentoxide: N = 15·33 %; P = 5·60 %.

Calculated for $C_6H_5NHNH_2H_2PO_4.C_4H_5(OH)_3C(N_2HC_6H_5)CHN_2HC_6H_5$; N = 15·38 %; P = 5·68 %.

The compound therefore appeared to be the phenylhydrazine salt of the osazone of hexosemonophosphoric acid. It is, however, not identical with the compound of the same empirical formula obtained by Young from hexose-diphosphoric acid, with elimination of one molecule of phosphoric acid, since the latter melted at 151°.

The chloroform extract from the crude osazone yielded, on evaporation, a small quantity of a crystalline compound readily soluble in ethyl alcohol, which, by recrystallisation from ethyl alcohol and chloroform, was obtained in yellow plates, melting with decomposition at 190°. The substance contained phosphorus and was readily soluble in dilute sodium hydroxide, though very sparingly soluble in water. Unfortunately the quantity was insufficient for analysis.

Hydrolysis of hexosemonophosphoric acid by acids.

Hexosemonophosphoric acid is very resistant to hydrolysis by acids at ordinary temperatures.

A solution of the acid in *N* sulphuric acid, after being kept for four months at room temperature contained only a trace of free phosphoric acid. When the solution of the acid is boiled either alone or with the addition of mineral acid, free phosphoric acid is slowly liberated and a reducing substance formed. Table III gives the data of an experiment in which 5·4 g. of hexosemonophosphoric acid prepared from glucose, was heated with 250 cc. of *N*/5 sulphuric acid at 97°, the rotation and free phosphoric acid being estimated from time to time. The total phosphorus = 0·643 g.

Table III.

Time of heating, hours	Rotation in 1 dm. tube	P present as free PO_4, g.	P present as free PO_4 in percentage of total P
0	+0·625°	0	0
1	+0·568°	—	—
3	+0·544°	—	—
16½	+0·507°	0·170	26·4
36	+0·427°	0·274	42·6
80	+0·327°	0·374	58·2

After 80 hours the solution had become dark brown in colour and the rotation was difficult to measure. The hydrolysis was accordingly stopped and the solution neutralised with baryta. The precipitate, which consisted entirely of barium phosphate and sulphate, was filtered off and the clear filtrate treated with an equal volume of alcohol, which precipitated the barium salt of the unchanged hexosemonophosphoric acid. The phosphorus content and specific rotation of this salt agreed with those of the original barium hexosemonophosphate. The alcoholic filtrate reduced Fehling's solution and contained only a trace of phosphorus. After distilling off the alcohol the reducing power of the solution was estimated by Bertrand's method and its rotation measured.

Reducing power calculated as glucose = 0·083 g. per 100 cc. equivalent to 0·98 for total original solution.

Rotation in 4 dm. tube = + 0·097°.

$$\frac{\text{Rotation in 4 dm. tube}}{\text{Reducing power as glucose in 100 cc.}} = 1{\cdot}17.$$ Calculated for glucose = 2·11.

The amount of sugar formed during the hydrolysis, calculated from the amount of phosphoric acid liberated is equal to 2·17 g. or more than twice the quantity actually estimated in the solution. It would seem therefore that a considerable proportion of the sugar is destroyed during the boiling. This solution yielded an osazone which, after being twice recrystallised from dilute pyridine, melted at 205°–206° and did not lower the melting point of glucosazone when intimately mixed with the latter.

In another experiment, Table IV, a solution of hexosemonophosphoric acid prepared from laevulose was boiled under a reflux condenser, the reducing power and the rotation being measured from time to time.

Table IV.

Time in hours	Rotation in 4 dm. tube	Reducing power as glucose in 100 cc.	$\frac{\text{Rotation}}{\text{Reducing power}}$
0	+1·339°	0·59	2·27
2	+1·064°	0·64	1·66
4	+1·024°	0·64	1·60
10	+0·896°	0·64	1·40
16	+0·788°	0·65	1·21

At the end of 16 hours' boiling the free phosphate amounted to 0·0795 g. P per 100 cc. or 53 % of the total phosphorus (0·1499 g. per 100 cc.). After precipitating the unchanged ester by basic lead acetate the filtrate was freed from lead by sulphuretted hydrogen and the reducing power etc. determined. The value found for the ratio $\frac{\text{Rotation in 4 dm. tube}}{\text{Reducing power as glucose in 100 cc.}}$ was equal to 0·90.

Hydrolysis of hexosemonophosphoric acid by emulsin.

Hexosemonophosphoric acid is hydrolysed by emulsin yielding also in this case, free phosphoric acid and a dextrorotatory substance from which glucosazone can be obtained.

A solution of 1·876 g. anhydrous barium hexosemonophosphate in 24 cc. water was treated with 2 cc. of an extract of emulsin[1] prepared from sweet almonds, and was incubated at 37° in presence of toluene. At intervals the rotation was measured, after filtering off the precipitated barium phosphate. Table V gives the results of these determinations. On the 10th day a further 1 cc. emulsin extract was added but the rotation has been calculated for the original volume of the solution.

Table V.

Time of incubation, days	Rotation in 2 dm. tube at 20°	Percentage of salt hydrolysed as measured by free PO_4
0	+1·680°	—
1	+1·579°	—
4	+1·376°	—
10	+1·334°	—
13	+1·339°	66

On the 13th day the remaining solution was neutralised to phenolphthalein with baryta and the total free phosphate estimated. The clear filtrate was treated with $1\frac{1}{2}$ volumes of alcohol and the precipitate filtered off, dried at 78° *in vacuo*, and examined.

Weight = 0·370 g. equivalent to 0·53 g. for the total original solution.

Found Ba = 32·8 %; P = 6·87 %; $[\alpha]_D^{20} = +11·3$ %.

[1] This extract contained a slight trace of reducing substance.

The substance probably consisted chiefly of unchanged barium hexosemonophosphate. The filtrate and washings from this precipitate were made up to 100 cc. They contained a reducing substance equivalent to 0·240 g. glucose as estimated by Bertrand's method. This amount is equivalent to 0·347 g. for the original solution.

Rotation in 4 dm. tube = + 0·350°.

$$\frac{\text{Rotation in 4 dm. tube}}{\text{Reducing power as glucose in 100 cc.}} = 1{\cdot}46.$$

On evaporation this solution yielded a syrup which could not be made to crystallise but with phenylhydrazine yielded an osazone. After recrystallisation from dilute pyridine this melted at 204°–205°, and did not lower the melting point of glucosazone when intimately mixed with the latter.

Hydrolysis of barium hexosediphosphate by emulsin.

The hydrolytic action of emulsin on hexosediphosphate was established by Harding [1912] who estimated the phosphoric acid set free but did not further examine the products.

In view of the possibility that the optically active substances produced under these conditions might differ from those produced by acid hydrolysis it was thought advisable to investigate this point.

1 g. of barium hexosediphosphate was incubated with 1 cc. emulsin extract and 10 cc. water at 37°, in presence of toluene. The salt only dissolved to a slight extent in the water. After 14 days the solution was filtered from the solid matter, which consisted of unchanged barium hexosediphosphate and barium phosphate, and examined polarimetrically.

$$\alpha^{18} \text{ in 1 dm. tube} = - 0{\cdot}530°.$$

Estimation of free and total phosphate in both precipitate and solution indicated that about 60 % of the phosphoric acid had been liberated. On neutralising the solution with baryta and adding alcohol a small amount of soluble barium salt was precipitated while the solution contained the laevorotatory substance.

It appears therefore that the hydrolysis of hexosediphosphoric acid by enzymes results in products similar to those obtained by acid hydrolysis. Further experiments are, however, being carried out.

The hexosemonophosphoric acid produced by partial hydrolysis of hexosediphosphoric acid.

A quantity of this ester was prepared by Neuberg's [1918] method in order to compare it with the compound obtained by the fermentation of sugar.

9·3 g. of crude barium salt was thus obtained from 19 g. barium hexosediphosphate by boiling the latter with a solution of 9·5 g. oxalic acid in 150 cc. water. After purification by repeatedly dissolving it in 10 % alcohol and precipitating the clear solution with alcohol, the product (5·3 g.) was dried at 78° *in vacuo* over phosphorus pentoxide and analysed.

Found Ba = 33·26 %; P = 7·58 %; $[\alpha]_D^{19}$ in 5·2% aqueous solution $= + 0{\cdot}38°$.

Calculated for $C_6H_{11}O_5PO_4BaH_2O$; Ba = 33·22 %; P = 7·51 %.

According to Neuberg the barium salt holds one molecule of water which is not given up at 105° and the above analyses are in agreement with this view.

The specific rotation of the acid, prepared from the solution of the barium salt by adding the calculated amount of oxalic acid was also determined:

$[\alpha]_D^{20}$ in 3·3 % aqueous solution $= + 1{\cdot}49°$.

With such small polarimetric readings, however, the possible error in the above values is relatively large. The solution of the acid was boiled with such excess of oxalic acid as brought the total acidity equal to *N*.

The solution rapidly became laevorotatory, as is shown in Table VI.

Table VI.

Time of boiling (hours)	Rotation in 2 dm. tube	Percentage of salt hydrolysed as measured by free phosphate
0	+0·094	0
$\frac{1}{2}$	−0·176	—
$1\frac{1}{2}$	−0·481	—
$4\frac{1}{2}$	−0·822	68

The behaviour of fermentation hexosemonophosphoric acid on hydrolysis is thus very different from that of hexosediphosphoric acid, and of the monophosphoric acid obtained from it by partial hydrolysis, both of which yield laevorotatory products. Table VII gives the results of experiments with each of the above compounds for comparison, the figures for the diphosphoric ester (Nos. 7–9) being those of Young [1909].

Table VII.

	Substance	Method of hydrolysis	Percentage of phosphate liberated	Rotation in 4 dm. tube: Before hydrolysis	Rotation in 4 dm. tube: After hydrolysis
1.	Hexosemonophosphoric acid from glucose	Solution of acid in *N*/5 H_2SO_4 at 97° 80 hours	58·2	+2·50°	+1·31°
2.	Do. Do.	Solution of acid boiled 27 hours	73	+0·374°	+0·234°
3.	Do. from fructose	„ „ 16 „	53	+1·339°	+0·788°
4.	Do. Do.	„ „ $10\frac{1}{2}$ „	42	+0·816°	+0·532°
5.	Barium hexosemonophosphate from glucose	Emulsin; 13 days at 37°	66	+1·680°*	+1·339°*
6.	Hexosemonophosphoric acid from hexosediphosphoric acid by partial hydrolysis (Neuberg's method)	Solution of acid in *N* oxalic acid boiled $4\frac{1}{2}$ hours	68	+0·094°	−0·822°
7.	Hexosediphosphoric acid from glucose (Young)	Solution of acid boiled 27 hours	91	—	—
8.	Do. Do.	„ „ 14 „	—	+0·161°	−0·658°
9.	Do. from fructose (Young)	„ „ 10 „	—	+0·416°	−1·514°
10.	Barium hexosediphosphate	Emulsin; 14 days at 37°	60	(+0·30† calc.) (salt not in solution)	−0°·530†

* 2 dm. tube. † 1 dm. tube.

It would seem improbable therefore that the hexosemonophosphoric acid found in the products of fermentation is formed from hexosediphosphoric acid by hydrolysis, enzymic or otherwise. It may conceivably be an intermediate stage in the formation of the diphosphoric ester and indeed its behaviour with yeast juice and zymin lends some weight to such a view. On the other hand it may be formed by the synthetic power of the yeast enzymes but have no part in the main cycle of reactions which constitutes alcoholic fermentation.

The analytical data so far obtained are all in agreement with the formula assigned to the compound, that of a hexosemonophosphoric ester, but it is admitted that further criteria of purity and homogeneity are desirable before this formula can be considered as established. The formation of an osazone retaining the phosphoric acid radicle indicates that the latter is not attached either to the terminal aldehydic carbon atom or that adjacent to it, but its position in the molecule cannot be definitely stated at present. The nature of the hexose is also undecided. The reducing substance produced on hydrolysis is dextrorotatory but less so than glucose. The ester itself whether produced from glucose or fructose gives Selivanov's reaction for fructose but not so strongly as an equivalent quantity of the latter sugar. These facts and the formation of glucosazone from the products of hydrolysis may perhaps indicate that the compound is a mixture of the monophosphoric esters of glucose and fructose. More cannot be said until further work has been done.

Summary.

1. When fructose or glucose is fermented by yeast juice in presence of suitable amounts of a soluble phosphate, an ester having the composition of a hexosemonophosphoric acid is formed in addition to the hexosediphosphoric acid described by Young, Ivanov and Lebedev.

2. A method is described for the separation of this new phosphoric ester from the other products of the fermentation and for the purification of its barium salt.

3. Hexosemonophosphoric acid is strongly dextrorotatory; $[\alpha]_D^{20} = +25{\cdot}0°$ in aqueous solution.

The metallic salts with the exception of the basic salts of the heavy metals, are all readily soluble in water and are amorphous. A crystalline brucine salt has been obtained.

4. The phenylhydrazine salt of the osazone of hexosemonophosphoric acid has been obtained. It is not identical with the compound of the same empirical formula prepared by Young and Lebedev from hexosediphosphoric acid.

5. On hydrolysis by acids or by emulsin, hexosemonophosphoric acid yields free phosphoric acid and a dextrorotatory reducing substance from which glucosazone has been obtained. The rotatory power of this product is, however, less than that of pure glucose.

6. By its specific rotation and its behaviour on hydrolysis the compound is sharply distinguished from the hexosemonophosphoric acid prepared by Neuberg by partial hydrolysis of hexosediphosphoric acid.

7. The alkali salts of hexosemonophosphoric acid are readily fermented by yeast juice and zymin.

8. It is improbable that hexosemonophosphoric acid is produced by the hydrolysis of the diphosphoric ester during fermentation. It may perhaps be an intermediate stage in the formation of the latter compound.

In conclusion I wish to thank Professor A. Harden for the interest which he has taken in this investigation.

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LXXXVI. MAMMARY SECRETION. IV.

THE RELATION OF PROTEIN TO OTHER DIETARY CONSTITUENTS.

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(*Received October 24th, 1922.*)

I. Introductory and Historical.

It is generally held that the diet of a lactating animal should contain a large amount of protein, and it seems quite obvious that a maximal growth of the young cannot be obtained if the mother's protein intake is low. On the other hand it has been demonstrated [Hartwell, 1921, 2] that large amounts of protein fed to the mother, result in harmful effects to the young. It has, however, been found [Hartwell, 1922] that large quantities of whole milk, and large amounts of yeast extract added to the mother's excess protein diet, prevent the bad symptoms in the litters. The obvious, though possibly not the correct explanation of such results, was that the important factor was vitamin B, since this is present in both milk and yeast. Also it was shown [Hartwell, 1922] that lactose, butter, milk-ash and calcium lactate were ineffective in improving the bad condition. Consequently the milk effect was not due to any obvious dietary factor.

The experiments described in this paper were started in the hope of throwing some light on the relation of vitamin B to protein in the metabolism of the lactating animal.

That there is a possible relation between vitamin B and protein is to be seen from the work of Karr [1920]. He found that the dog refused food unless vitamin B was included in the diet, and since this animal is a carnivore, it may be that the vitamin is in some way essential for protein metabolism.

In the experiments to be detailed here the mother rats were fed on an excess protein diet, to which were added various fruit and vegetable juices and extracts made from other foods said to contain vitamin B. From the results described, it appears that the addition to the mother's protein-rich diet of juices of, or extracts from, foods reported to contain vitamin B, is effective in rendering the nature of the milk more normal.

At the present time, the published results as to presence or absence of vitamins in foods show discrepancies. This is probably due to

(1) The different methods used for testing the presence of the vitamins.

(2) The lack of quantitative estimations.

It appears to be generally accepted that vitamin B is widely distributed in the plant kingdom, although there are differences of opinion as to the amount present.

Osborne and Mendel [1920, 2] state that the edible part of an orange contains vitamin B and that the potency is similar to that of comparable volumes of cow's milk. Grape juice they consider to be less potent than orange; apples contain the vitamin, but are not rich in it. These observers [1920, 1] also find that tomatoes and carrots contain appreciable amounts of the water-soluble vitamin; potato is quite a good source, but not so good as tomato. Beetroot contains less of the substance. The occurrence of vitamin B in animal tissues is possibly less extensive, but it may be present in smaller quantities. Cooper [1913] prepared a curative solution of the antineuritic substance from horse flesh and from beef.

According to Drummond [1919] animal tissues (with one or two exceptions) are deficient in the water-soluble antineuritic factor. This observer fed young rats on protein-free extracts of muscle from herring, cod and salmon and found that his solutions contained no vitamin B. When the extract was made from the whole herring, he obtained a slight growth and concludes that the source of the vitamin in this case was the ova and glands, shown to contain the antineuritic vitamin by Chick and Hume [1917]. Drummond does not explain how he prepared the protein-free solutions and it is possible that any vitamin present was adsorbed when the protein was precipitated. If the factor investigated in the experiments below be vitamin B the results obtained contradict those of Drummond.

II. Methods.

The technique employed has been fully discussed in a previous paper [Hartwell, 1921, 1]. At least three mother rats were fed on the various diets and each attempted to rear six baby rats. One typical curve is shown as representative of the three. In some experiments where two or three members of one litter survived and another litter all died (*e.g.* cucumber juice, Fig. 1) two growth curves are given as showing the best and worst growth on the given diet.

The basal diet was:

(i) *White bread,*

(ii) *Butter for fat and vitamin A,*

(iii) *Lemon juice for vitamin C,*

(iv) *Caseinogen*
(v) *Salt mixture* } [Hartwell, 1922],

to which was added one of the following as potential source of vitamin B:

(*a*) *Fruit or vegetable juices.* These were made as follows: the soft fruits were placed in muslin and the juice squeezed out by hand. The harder vegetables (*e.g.* potato, carrot) were grated and then squashed in a hand press. The juice was filtered through muslin and allowed to stand in a cylinder so that the starch (if present) settled at the bottom of the vessel and the juice was decanted. All juice was freshly prepared, just before being fed to the animals.

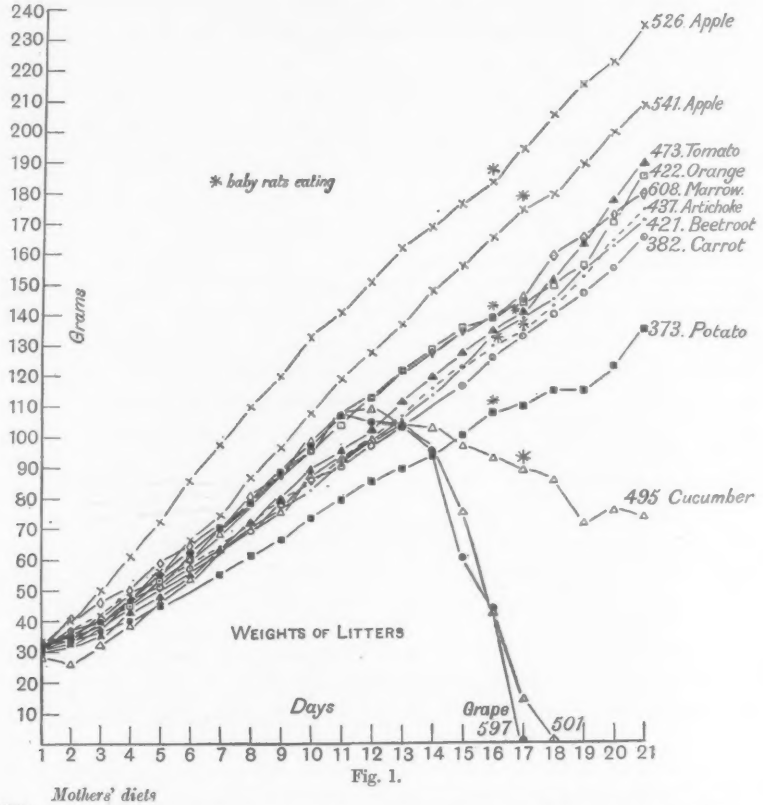

Fig. 1.

Mothers' diets

526 } 541 }	× —— ×	15 g. bread, 5 g. caseinogen, 0·5 g. butter, 0·7 cc. lemon juice, 50 cc. apple juice				
473	▲ —— ▲	,,	,,	,,	,,	50 cc. tomato juice
422	□ —— □	,,	,,	,,	,,	50 cc. orange ,,
437	• - - •	,,	,,	,,	,,	50 cc. artichoke ,,
421	• —— •	,,	,,	,,	,,	50 cc. beetroot ,,
382	○ —— ○	,,	,,	,,	,,	50 cc. carrot ,,
373	■ —— ■	,,	,,	,,	,,	50 cc. potato ,,
608	◊ —— ◊	,,	,,	,,	,,	50 cc. marrow ,,
495 } 501 }	△ —— △	,,	,,	,,	,,	50 cc. cucumber ,,
597	● —— ●	,,	,,	,,	,,	50 cc. grape ,,

(*b*) *Meat or fish extracts.* The meat was freed from fat as far as possible and minced finely. The fish muscle was removed from the bones (in the herring the backbone was taken out, but probably a few small bones were left) and minced. No roe was included.

The minced meat and fish were weighed, placed in a bottle, warm distilled water added (1 cc. water to 1 g. flesh) and the whole shaken on a mechanical shaker for 1½ hours. The contents of the bottle were then transferred to a flask, which was placed in a boiling water-bath for some of the protein in the solution to coagulate. The whole was filtered through paper and the filtrate fed to the animals.

(*c*) *Wheat germ extract.* The wheat germ was added to warm distilled water in the proportion of 3 g. wheat to 50 cc. water. This mixture was shaken for 1½ hours and then filtered through paper. The volume of the filtrate was measured and the liquid boiled to coagulate some of the protein. When cold the volume was made up with distilled water to what it was before boiling and the protein filtered off. This filtrate was fed to the animals.

(*d*) *Extract rich in vitamin B made from crude "marmite."* 250 g. "marmite" were dissolved in 400 cc. distilled water and transferred to a Winchester quart bottle. 1600 cc. absolute alcohol were added and the whole shaken for 3 hours. This liquid was decanted (filtered if necessary) and distilled *in vacuo* at 40°–50° until all the alcohol had been distilled off. The remainder was left to cool, filtered, and the volume made up to 250 cc. with distilled water. It was boiled for 4 hours to sterilise.

(*e*) *Egg yolk solution.* The egg yolk was separated from the white as much as possible, 50 cc. warm distilled water added and the whole well mixed.

The diet given.

In all the experiments to be described in this paper the proportion of white bread to protein was the same as that previously used as an excess protein diet, *i.e.* 15 g. bread; 5 g. protein (caseinogen).

The initial basal ration consisted of: 15 g. bread, 5 g. protein (caseinogen), 0·7 g. salt mixture, 0·5 g. butter, 0·7 cc. lemon juice.

To this were added 50 cc. of fruit or vegetable juice, meat extract, or egg yolk solution etc.

When wheat germ or soya bean was given as solids, 3 g. were added to the above ration and water was used to mix the dry constituents.

All the constituents were increased proportionately when necessary.

III. Results.

(i) *Vegetable and fruit juices.*

Jerusalem artichoke, carrot, tomato and potato gave excellent results. The baby rats were normal in all respects and were extraordinarily fit and healthy. The litters belonging to the mothers which were given tomato juice in their

diets, were, perhaps, just fitter than the others and had very thick coats. Representative growth curves are shown in Fig. 1, 437, 382, 473, 373.

Apple, orange, beetroot and vegetable marrow juices were also effective in preventing very bad symptoms in the young, but the babies were not so healthy and fit as were those whose mothers received artichoke, carrot, tomato or potato juice, although the "apple babies" grew at a greater rate.

In all these experiments slight spasms were seen in the young, but the condition of the babies was never very bad. The spasms were generally noticed for one day only, and the growth curve was not interfered with, *i.e.* no loss of weight was observed. In the case of the "apple babies" the rate of growth was practically maximal. From the 18th–20th day screaming fits [Hartwell, 1921, 2] were noticed, but in spite of this the babies seemed well and gained weight. All were successfully weaned at the 21st day (Fig. 1, 526, 541, 422, 421, 608).

Cucumber juice when fed to the mother was of no effect in relieving the bad symptoms in the young. All three litters suffered badly, the spasms were severe and only 4 out of the 18 young survived. These were weakly for about two weeks after weaning (Fig. 1, 495, 501).

Grape juice, gave even worse results than cucumber juice. All three litters suffered badly and none of the young survived (Fig. 1, 597).

(ii) *Wheat germ and soya bean.*

These two foods were tried because both are reported to be rich in vitamin B.

(*a*) *Wheat germ* 3·0 *g. and wheat germ extract* 50 *cc.* (preparation see p. 828) were most effective. The litters did well and showed none of the bad symptoms. In the first series of experiments, it is interesting to note that the mother was receiving still more protein, since wheat germ itself contains 24·3 % of protein [Plimmer, 1921] (Fig. 2, 405, 480).

(*b*) *Soya bean.* When 3 g. were added to the mother's basal diet the litters suffered severely; all the babies had bad spasms; 11 out of 16 survived, but were weakly for some time after weaning.

Soya bean contains 33·7 % protein [Plimmer, 1921] and therefore it seemed possible that the bad results of the above experiments might be explained by the increase of protein in the mother's diet. Accordingly three more experiments were tried in which the soya bean was increased from 3 to 6 g. in proportion to the 15 g. bread, 5 g. protein etc. In this case the babies all survived and were practically normal. Slight spasms were noticed, but only for one day. The results were comparable to those obtained when the mother received apple juice (Fig. 3).

(iii) *Fish and meat extracts. Egg yolk. Whey.*

Lean muscle (*beef*) *and herring* extracts when added to the mother's diet entirely protected the young from any harmful effects (Fig. 4, 390, 433).

Cod extract was nearly as good, but not quite. No spasms were noticed, but the baby rats were not quite so lively as normal animals. However, three days after weaning (on a bread and whole milk diet) they had recovered entirely (Fig. 4, 447).

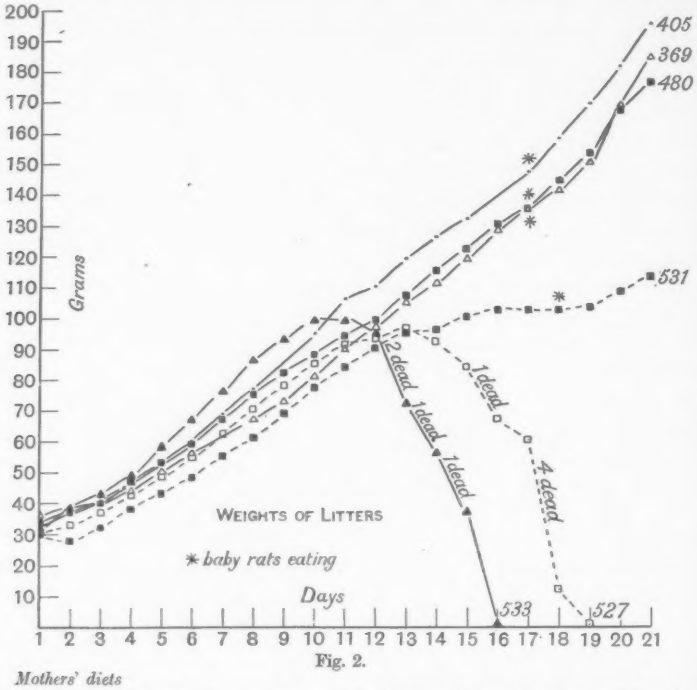

Fig. 2.

Mothers' diets

405 • —— • 15 g. bread, 5 g. caseinogen, 0·5 g. butter, 0·7 g. salt mixture, 0·7 cc. lemon juice, *3·0 g. wheat germ.*

480 ■ —— ■ 15 b., 5 c., 0·5 b., 0·7 salt, 0·7 lemon (see above), *50 cc. wheat germ extract.*

369 △ —— △ " " " *3 cc. extract from marmite.*

531 ■ – – ■ } 15 b., 5 c., 0·5 b., 0·7 salt, 0·7 lemon (see above), *50 cc. wheat extract previously*

527 □ – – □ } *boiled with charcoal.*

533 ▲ —— ▲ 15 b., 5 c., 0·5 b., 0·7 salt, 0·7 lemon (see above), *50 cc. marmite extract previously boiled with charcoal.*

Egg yolk was extraordinarily good. The growth curve of the young was equal to that obtained on any diet (*i.e.* maximal) and the babies were absolutely fit in all respects. Their coats were especially thick and long. This is interesting because egg yolk itself contains 15 % of protein (Fig. 4, 464).

Whey. It has previously been shown that the whey from 100 cc. whole milk when added to the mother's excess protein diet was adequate in safeguarding the young. In these experiments 50 cc. were tried in order to compare these results with those obtained when the mothers received 50 cc. of fruit juice, etc.

The babies suffered badly; 13 out of 24 survived, but these were miserable specimens. They were weakly and their coats were thin and dirty (a typical sign that an animal is not fit). They were put on a bread and milk diet on weaning, but it was over a fortnight before they could be considered normal (Fig. 4, 515).

(iv) *Extract rich in vitamin B made from crude "marmite."*

(Preparation given on p. 827.)

3 cc. of the extract were added to the basal ration, and proved most effective. All three litters did well and were normal in all respects (Fig. 2, 369).

The results of the above experiments are tabulated on p. 832. The distribution of vitamin B as given in the report of the Medical Research Council [1919] and by Eddy [1921] is included.

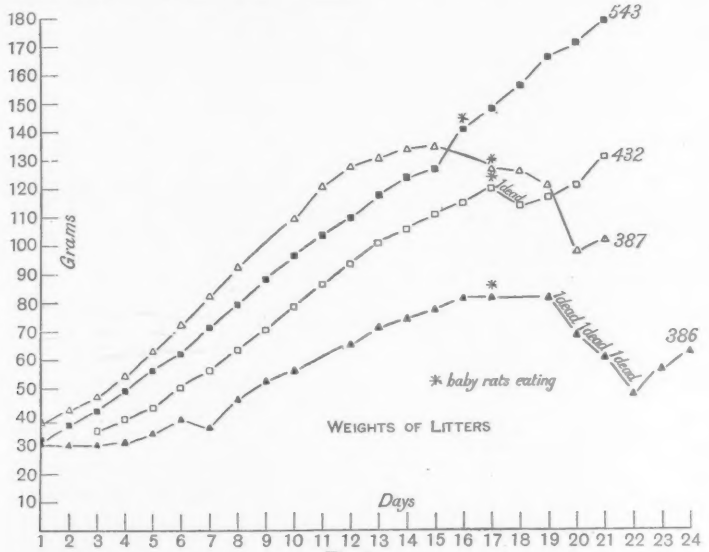

Fig. 3.

Mothers' diets

543 ■ —— ■ } 15 g. bread, 5 g. caseinogen, 0·5 g. butter, 0·7 g. salt mixture, 0·7 cc. lemon
432 □ —— □ } juice, *6 g. soya bean.*
386 ▲ —— ▲ } 15 g. bread, 5 g. caseinogen, 0·5 g. butter, 0·7 g. salt mixture, 0·7 cc. lemon
387 △ —— △ } juice, *3 g. soya bean.*

(v) *Experiments with a diet physiologically incomplete.*

The rats were fed on a diet of bread and caseinogen, as before, in the proportion of 15 : 5 and to this was added tomato juice, wheat germ or marmite extract. Such diets were incomplete physiologically, since salt mixture and fat were omitted. Also vitamin A would be poorly represented, if present at all. As explained in a previous paper [Hartwell, 1922] the

caseinogen used was not pure and might therefore contain a small amount of vitamin A, as would also the wheat germ and tomato juice.

Three mothers were fed on each diet and in all nine experiments they remained fit and well. The young had a good growth curve and no spasms were seen. The baby rats appeared perfectly fit and healthy, and were quite equal to those whose mothers were getting a more complete diet (Fig. 5, 396, 519, 553).

Fruit and vegetables.

50 cc. juice	M.R.C. on 3 + system	Eddy on 4 + system	Condition of litters
Apple[1]	Not given	+ +	Slight spasms. Growth good
Artichoke	,,	Not given	No spasms. Growth good
Beetroot[2]	,,	+	Spasms. Babies weakly, but all 18 survived
Carrot[3]	+	+ + +	No spasms. Growth good
Cucumber	Not given	Not given	Bad spasms. 4 out of 18 young survived
Grapes[4]	,,	+	Bad spasms, all young died
Orange[1]	,,	+ + +	Slight spasms. Growth good
Potato	+	+ + +	No spasms. Slight jerky type of walking. Growth good
Soya bean (3 g.)	+ +	+ + +	Bad spasms, 11 out of 16 young survived, but were weakly
,, ,, (6 g.)	+ +	+ + +	Very slight spasms. Babies normal and healthy
Tomato[2]	Not given	+ + +	No spasms. Growth good. Babies coats very thick. Young *very* fit
Vegetable marrow	,,	Not given	Slight spasms, but quite good growth
Wheat germ[5] 3 g.	+ + +	+ + +	No spasms. Very good growth
Wheat germ extract	—	—	,, ,, ,,
Yeast 3 g.[5]	+ + +	+ + + +	,, ,, ,,
Yeast extract "marmite"	+ + +	+ + +	,, ,, ,,

Meat, fish, eggs, milk.

Cod[6]	White fish very slight if any	+	No spasms. Good growth. Not *quite* fit
Egg yolk[6]	Eggs + + +	Eggs + +	No spasms. *Very* good growth, extraordinarily fit babies
Herring	Very slight if any	+ +	No spasms. Quite good growth
Meat extract[5] 1 g. 1 cc. water	Lean meat +	0	No spasms. Good growth
Whey	Milk +	+ + +	Bad spasms. 13 out of 24 young survived, but all were weakly

[1] Osborne and Mendel [1920, 2].
[2] —— —— [1919].
[3] Sugiura and Benedict [1918].
Denton and Kohman [1918].
[4] Osborne and Mendel [1920, 1]. (Commercial grape juice.)
[5] Chick and Hume [1917, 1, 2].
[6] Cooper [1913].

From such a series of experiments it seems justifiable to conclude that protein and the substance supplied in tomato, marmite, wheat germ etc. (? vitamin B) are of primary importance in the diet of a lactating animal.

(vi) *Experiments demonstrating adsorption by charcoal of the accessory factor.*

From the experiments just described it is clear that the protective substance has a distribution similar to that of vitamin B. It has been shown by Chamberlain and Vedder [1911] that the active principle (prepared from rice

polishings) which prevents polyneuritis in fowls is adsorbed by filtering through animal charcoal.

Cooper [1913] found that a curative solution prepared from horse flesh lost its activity to the extent of 30 % when filtered six times through a bed of animal charcoal. It was therefore decided to try if the protective substance shared this property of vitamin B. Accordingly the following experiments were made in which the mother rats were fed on marmite and wheat germ extracts which had previously been boiled with animal charcoal.

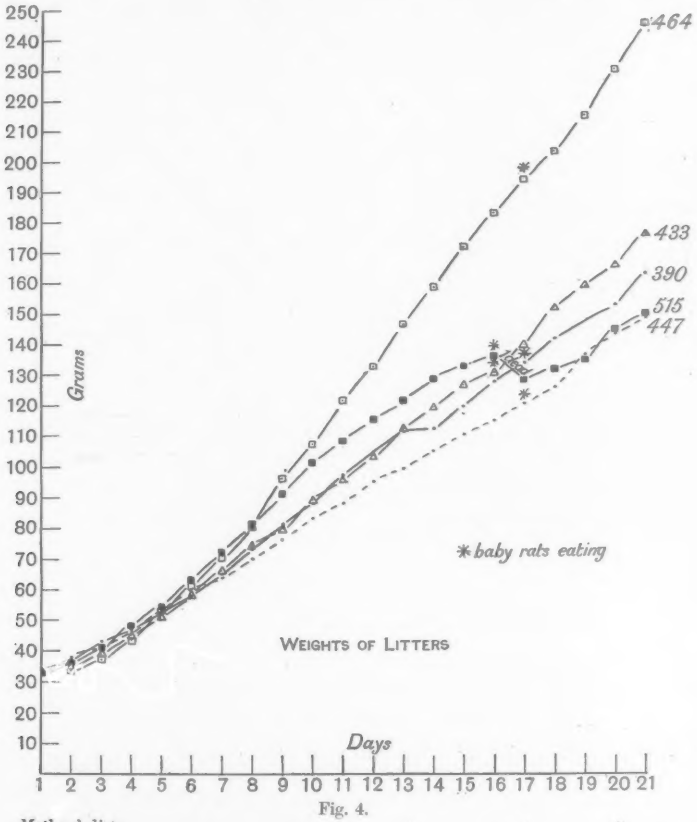

Fig. 4.

Mothers' diets

464 □ —— □ 15 g. bread, 5 g. caseinogen, 0·5 g. butter, 0·7 g. salt mixture, 0·7 cc. lemon juice, *50 cc. egg yolk solution.*

433 △ —— △ 15 b., 5 c., 0·5 b., 0·7 salt, 0·7 lemon (see above), *50 cc. herring extract.*

390 • —— • „ „ „ „ *50 cc. meat extract.*

515 ■ —— ■ „ „ „ „ *50 cc. whey.*

447 • – – • „ „ „ „ *50 cc. cod extract.*

Preparation of extracts.

(*a*) *Wheat germ.* The extract was prepared as previously described (p. 828). Animal charcoal was added and the whole boiled. When boiling, a little of the liquid was taken in a spoon; if the charcoal settled rapidly and left a clear liquid on top, the whole was allowed to cool. If the charcoal remained suspended in the liquid, more charcoal was added and the boiling continued until the clear liquid settled out when tested in the spoon. When cool the volume was made up with distilled water to what it was before boiling, and the whole filtered. This clear filtrate was added to the diet.

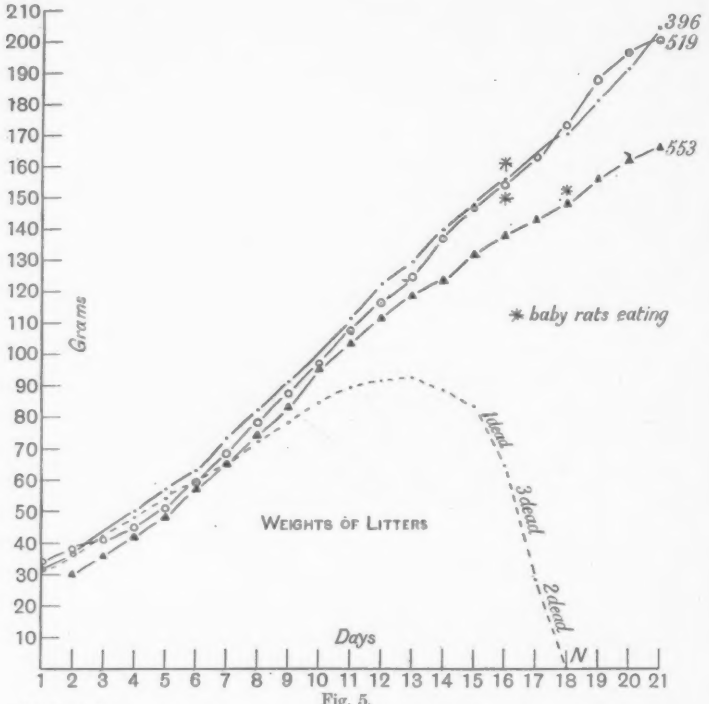

Fig. 5.

Mothers' diets

396 • —— • 15 g. bread, 5 g. caseinogen, *3 cc. extract from marmite.*
519 ○ —— ○ ,, ,, *3 g. wheat germ.*
553 ▲ —— ▲ ,, ,, *50 cc. tomato juice.*
N • – – • ,, ,, 50 cc. water (taken from Hartwell (1921, 1)) for comparison.

(*b*) "*Marmite*" (*yeast extract*). 6 % marmite solution was made with hot water. This was boiled with charcoal (as described above), cooled, the volume made up and filtered. This process was repeated (usually three times) until a clear, colourless filtrate was obtained. This was used for the feeding.

The diet was the same as that used in the preceding experiments, *i.e.* 15 g. bread, 5 g. protein, 0·5 g. butter, 0·7 g. salt mixture, 0·7 cc. lemon juice + 50 cc. of one of the above extracts.

Three experiments were made with each diet.

Results. When the mothers received the marmite extract which had been boiled with animal charcoal, the babies suffered badly and none survived. A typical curve is shown in Fig. 2 with the yeast "charcoal" extract, two litters died and one survived, though all had bad spasms. The young which survived were weakly and were not normal for some time after weaning.

The slight difference in these results may be accounted for by the fact that the marmite extract was boiled three times with charcoal and the wheat extract, only once, so that in the former case there might be more complete adsorption (Fig. 2, 527, 531, 533).

IV. Comments.

Many natural foods or extracts made from them, contain a factor which protects the suckling rats from the harmful effects of excess of protein in the mother rat's diet.

The distribution of this factor is similar to that of vitamin B. The foods reported to be especially rich in this vitamin, *e.g.* egg yolk, wheat germ, when added to the mother's diet not only prevent the bad symptoms, but provide for good growth in the young. Grape juice when fed to the mother was of no effect in relieving the symptoms in the baby rats; all the 18 young (3 litters) died. According to Eddy the vitamin B content of grapes is represented by + (see Table, p. 832).

There are, however, discrepancies between my findings, the work of other physiologists, and the tables published by the Medical Research Council and Eddy.

Drummond [1919] finds that there are no appreciable amounts of the water-soluble factor in the muscles of cod and herring. Eddy gives the vitamin B content of cod as + and herring + +. According to the experiments described in this paper, the extracts from cod and herring muscle had appreciable protective properties, since no spasms were seen in the young, when such extracts were added to the mother rat's diet.

Again Eddy represents orange and tomato as equal and containing + + + and apple as + +. My experiments show tomato to be very superior to orange and that apple and orange are about equal. When the mothers received a ration of 50 cc. orange juice per day, the litters developed spasms, while a ration of 25 cc. tomato juice added to the mother's diet was sufficient to give complete protection to the young.

Soya bean gave interesting results (Eddy + + +, M.R.C. Report + +). When 3 g. were fed to the mother rats, the babies developed spasms and were in a very poor condition; but with 6 g. instead of 3 g., the litters

were practically normal, only slight spasms were noticed and the growth was good. Soya bean contains 33·7 % of protein and hence adding 3 g. soya bean to the mothers' diet also increased the protein ration. With 6 g., however, the vitamin content of the diet was nearly doubled, while the percentage of protein was very little different from that of the diet with 3 g. protein. On the whole my results agree more closely with those of Eddy than with those published in the Medical Research Council's Report.

It is possible that the disagreement may be due to:

1. Method of investigation—some observers find the curative dose, while others merely add a definite amount of the food under investigation to a diet deficient in vitamin B.

2. The method of preparation of extracts used, since vitamins easily form adsorption compounds.

Further the protective factor is similar to vitamin B in its solubility and its property of adhering to charcoal. Consequently there is presumptive evidence that the protective factor is vitamin B. This is interesting as vitamin B is thus definitely linked with protein metabolism; the one gland that synthesises protein in large amount functions badly in the absence of vitamin B when there is excess of amino acids in the blood. The lactating animal, when there is excess of protein in the diet, needs much more vitamin B than normal, not for herself, but to regulate the activity of the mammary glands.

Summary.

1. When 50 cc. tomato, artichoke or carrot juices are added to the mother's basal high protein diet, the litters do well and show no harmful effects, such as would otherwise be produced by such a diet.

3 g. wheat germ or 50 cc. wheat germ extract give equally good results. Soya bean, 3 g., are ineffective, but 6 g. added to the above diet greatly improve the condition of the litters.

2. Apple and orange juices give good results, but not quite equal to those obtained with potato etc. The litters show good growth, but slight spasms are noticed. These do not last long and the growth curve is not impaired.

3. Wheat germ extract and marmite extract after being boiled with animal charcoal have no beneficial effect.

4. Beef muscle, herring, and cod extracts, when added to the mother's diet have also the property of protecting the young from any bad effects. Egg yolk has a similar protective action and the growth curve of the young is exceptionally good.

5. The substance in foods which exercises this protective action resembles vitamin B in its distribution, its solubility and the ease with which it is adsorbed by charcoal.

I wish to thank Prof. V. H. Mottram for his criticism and advice. Thanks are also due to the Medical Research Council for a grant which defrayed the expenses of this research.

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CAMBRIDGE: PRINTED BY J. B. PEACE, M.A., AT THE UNIVERSITY PRESS

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